A BOVINE MODEL TO STUDY REPRODUCTIVE AGING

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By

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Keywords: aging, bovine model, oocyte, ovary, reproduction

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ABSTRACT

Decline in fertility with age has been well documented in women. There are ethical limitations to use humans as a model for basic research, and there is a lack of well characterized animal model. The objective was to characterize and validate a bovine model for the study of age-associated subfertility. All experiments were conducted on the same group of 13-14 year old cows (n=10), and their 1-4 year old young daughters (n=10). Mother-daughter pairs were used to reduce genetic variations.

Follicular wave pattern in a natural reproductive cycle was maintained in old cows similar to that in daughters. We hypothesized that aging in cattle is associated with elevated circulating concentrations of FSH, and reduced concentrations of steroid hormones. As stated, circulating FSH concentrations were higher (P=0.009) during follicular waves in old than young cows. The ovulatory follicle in 2-wave cycles was smaller in old cows (P=0.04), but plasma estradiol concentrations were higher (P=0.01). Luteal phase progesterone tended to be lower in old than young cows (P=0.1). The number of 4-5 mm follicles recruited into a follicular wave was lower ($P<0.05$) in old cows than in their daughters.

The response to ovarian synchronization and superstimulatory treatments was compared between old and young cows. We hypothesized that aging in cattle is associated with decreased synchrony of an induced follicular wave after steroid treatment. Conversely, the emergence of an induced follicular wave was synchronous between age groups. The preovulatory LH surge was delayed in old compared to young cows (P=0.01), but the detected ovulation times were not different. Old cows had fewer ($P<0.01$) follicles ≥ 6 mm after superstimulation, and tended (P=0.1) to have fewer ovulations than their daughters (32 ± 4 versus 40 ± 3, respectively). The response of individual cows to successive superstimulatory treatments was correlated ($r>0.8$; $P<0.0001$).

The hypothesis of reduced oocyte developmental competence in old cows was tested by comparing embryo production and pregnancy rates between old and young cows. Fewer ($P=0.04$) embryos were recovered from old cows (6±2) than their daughters (12±2). A higher proportion ($P<0.01$) of unfertilized oocytes and/or uncleaved zygotes were recovered from old cows (222/312, 71%) than their daughters (119/316, 38%). The recovery of fewer embryos in old cows suggests reduced oocyte developmental competence. The survival of embryos after transfer into unrelated young recipients was similar between age groups.

The effects of advanced age on oocyte meiotic maturation and oocyte chromosome numbers abnormalities were studied in old and young cows. Our hypothesis of compromised oocyte meiotic maturation with age was not supported; similar or higher proportion of metaphase II oocytes were recovered from old than young cows. The abnormalities of oocyte chromosomal numbers were similar between age groups.

To conclude, endocrine, follicular and oocyte developmental changes in old cows are consistent with those reported for women approaching menopause. Therefore, our results validated the use of a bovine model to study age-associated subfertility in women. Unlike women, we did not detect an age-related increase in abnormalities of oocyte chromosome numbers in cattle.

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DEDICATION

I dedicate this thesis to Punjab Agricultural University, Ludhiana, India and to University of Saskatchewan, Canada for providing me the opportunities of quality education. I owe my success to these institutions while failures are my own shortcomings.

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LIST OF ABREVIATIONS

AI	artificial insemination
ART	assisted reproductive technology
C	celsius
CIDR	controlled internal drug release
CL	corpus luteum
COC	cumulus oocyte complex
ET	embryo transfer
FSH	follicle stimulating hormone
g	gram
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hours
im	intramuscular
IOI	interovulatory interval
IVF	in vitro fertilization
IWI	interwave interval
kg	kilogram
LH	luteinizing hormone
pg	picogram
MHz	megahertz
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
ng	nanogram
SAS	statistical analysis system
SEM	standard error of the mean
USP	United States pharmacopeia
VCD	4-Vinylcyclohexene diepoxide
vs.	versus
µg	microgram
µL	microlitre
µm	micron

ASRM	American Society for Reproductive Medicine
IETS	International Embryo Transfer Society
NIAMDD	National Institute of Arthritis, Metabolism, and Digestive Diseases
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIH	National Institute of Health
USDA	United States Department of Agriculture

CHAPTER 1

GENERAL INTRODUCTION

1.1 Age and Fertility

Demographic analyses of monogamous human populations that did not practice contraception revealed that fertility in women decreased with age (Tietze, 1957; Menken et al., 1986). However, these findings were confounded by decreased sexual activity with age, reproductive pathology associated with multiparity, and male infertility (Menken et al., 1986). These confounding variables were abrogated in a later retrospective study of 2193 nulliparous women whose husbands were sterile and who were artificially inseminated for 12 consecutive menstrual cycles using semen from fertile donors (Schwartz and Mayaux, 1982). Pregnancy rate in the women over 35 years of age was significantly lower (54%) than in the women below 31 years of age (74%). The results of more recent demographic studies (Chandra et al., 2005) substantiate the phenomenon of an age-related decline in female fertility. Furthermore, an increasing number of North American women delay childbearing until after 30 years of age (ASRM, 2004).

1.2 Assisted Reproductive Technologies

The success rates of assisted reproductive technologies in women decreased with age (Wright et al., 2006). In human IVF, 1-5 embryos (cleavage or blastocyst stage) are commonly transferred into the uterus of female patients to improve the chances of a successful live birth (Little et al., 2006). Patients over 35 years of age are usually transplanted with more embryos than younger patients (ASRM, 2006). Thus, multiple

births are very common in women using IVF; one of two babies born in United States using IVF was part of multiple births (Little et al., 2006). A major proportion of twins (33%) and triplets (40%) born were also conceived through IVF (Little et al., 2006). The average cost of a triplet birth is at least ten times more than a singleton birth (Little et al., 2006). The cost of nondonor ART per live birth was 3-4 times more in women over 40 years as compared to women below 30 years of age (Chambers et al., 2006).

1.3 Age-Associated Follicular and Endocrine Changes in Women

1.3.1 Natural Cycle

Endocrine studies in older women demonstrated higher circulating concentrations of FSH compared to younger women (Klein et al., 1996a; Soules et al., 2001), attributed to reduced negative feedback as a result of lower circulating concentrations of inhibin B (Klein et al., 2004). Steroid hormone concentrations in women did not show a consistent pattern but eventually decreased with advanced age (Klein et al., 1996a; te Velde et al., 1998; Soules et al., 2001). Reproductive aging in women has also been associated with a shortened follicular phase and thus, a shortened menstrual cycle (Klein et al., 1996a; Klein et al., 2002). The exact cause-and-effect relationships between endocrine and follicular changes and their impact on fertility remain unclear.

1.3.2 Assisted Reproductive Cycle

Older women using assisted reproductive technologies to become pregnant have a lower ovarian follicular response to gonadotropin stimulation than younger women, and embryos derived from oocytes of older women had lower implantation rates after transfer (ASRM, 2004). Studies involving oocyte donation from younger to older women are supportive of the notion that the age-related decline in fertility is due to reduced

developmental competence of oocytes, and not differences in uterine receptivity in women of advanced age (Sauer, 1998).

1.4 Issues

Oocyte chromosomal abnormalities, spindle defects and reduced mitochondrial function are implicated in the age-related decline in fertility (Pellestor et al., 2003; Baird et al., 2005), but the mechanisms are not well understood. Research progress has been limited in humans because of fewer developmentally competent oocytes available for hypothesis-based interventional studies, and most of the previous observations were made on oocytes that failed to develop into embryos after assisted reproductive cycles. Moreover, there is a lack of a well characterized animal model to study oocyte associated infertility in women over 35 years of age, and to determine ways to improve fertility in this age group.

1.5 An Ideal Animal Model

An ideal animal model to study reproductive aging in women should have the following characteristics: 1) close phylogenetic relationship to humans, 2) long reproductive life span, 3) well described reproductive physiology with mechanisms similar to that described in women, 4) age-related decline in fertility as reported in women of advanced age, 5) well developed reproductive and molecular technologies as well as the ability to manipulate follicular development, 6) relatively fewer ethical issues for conducting hypothesis-based observational or interventional studies, 7) economical, 8) easy availability of older individuals, 9) ease of animal handling and data collection.

1.6 Animal Models for the Study of Infertility in Women

1.6.1 Primates

The use of nonhuman primates especially rhesus monkey as a model to study reproductive and skeletal changes during menopause has been examined comprehensively (Black and Lane, 2002; Bellino and Wise, 2003; Nichols et al., 2005). It was concluded that the hormonal changes, irregularity of menstrual cycles, age-associated decline in fertility and ovarian follicle depletion have been observed in nonhuman primates during menopausal transition, and the changes appear to be similar to those observed in women of advanced age (Schramm et al., 2002; Bellino and Wise, 2003; Nichols et al., 2005). However, authors suggested that more studies are required to validate this model; the number of older animals available for research are also limited (Bellino and Wise, 2003). Furthermore, there is a lack of fundamental knowledge about ovarian follicular development in nonhuman primates.

1.6.2 Mouse

Unilateral ovariectomy, senescence accelerated, gene knockout (e.g. FSH receptor haploinsufficient mouse) and chemical (4-Vinylcyclohexene diepoxide or VCD)-induced models of aging are being investigated for the study of reproductive aging in women (Yuan et al., 2005; Danilovich and Ram Sairam, 2006). A rise in circulating concentrations of FSH, reduced estradiol concentrations, and depletion of primordial follicle pool has been observed in all models (Danilovich and Ram Sairam, 2006). Historically, mouse models are preferred based on easy availability of genetically uniform inbred lines, low cost, shorter life span, and ease of producing loss or gain of function genetic mutations. However, the basic ovarian follicular dynamics is not yet described in this species. Interestingly, most of the aging research in general is carried out

on C57BL/6 strain of US National Institute of Aging (Austad, 2003) or in other words, only one genotype is being used for most of the aging research.

1.6.3 Horse

Recent comparisons of ovarian follicular wave dynamics and circulating concentrations of gonadotropins and steroid hormones between mare and women (Ginther et al., 2004; Ginther et al., 2005) documented fundamental similarities between these two species. Both mare and women develop major follicular waves (characterized by the development of a dominant follicle) as well as minor follicular waves (largest follicle does not grow up to the size of a dominant follicle) during their estrous and menstrual cycles, respectively (Ginther et al., 2004). The incidence of major anovulatory follicular waves in horses was similar to that in women (Ginther et al., 2004). The diameter of the dominant follicle in mares was about two times larger than in women and this ratio was maintained from follicle selection to ovulation (Ginther et al., 2004). Based on these similarities, the mare is considered as a potential model species to study folliculogenesis in women. However, reproductive techniques (i.e., embryo transfer, in vitro fertilization and culture) are not very successful for this species, and it is difficult to precisely control ovarian follicular development.

1.6.4 Cattle

We proposed a bovine model to study ovarian function and the age-associated decline in fertility in women (Adams and Pierson, 1995; Malhi et al., 2005). Ovarian follicular wave development in cattle has been characterized over the last two decades (Pierson and Ginther, 1984; Pierson and Ginther, 1987; Ginther et al., 1989a; Adams et al., 1992b; Singh et al., 1997; Adams, 1998; Singh et al., 1998; Adams, 1999). Follicular wave has been defined as the synchronous growth of a group of follicles stimulated by a

surge of FSH (Adams et al., 1992b). The follicular waves in cattle are characterized by selection of one dominant follicle which continues to grow while others (subordinates) regress (Adams et al., 1993; Adams, 1999; Ginther, 2000). Dominant follicles of waves occurring during the luteal phase are anovulatory as a result of suppression of circulating LH by progesterone (Adams, 1999). Demise of the previous dominant follicle, in turn, permits the emergence of a new follicular wave, and the pattern repeats itself (Adams et al., 1992a; Adams, 1999). During luteolysis, decreasing concentrations of progesterone relieve suppression of LH release from the pituitary, and LH pulse-frequency increases. In response, the extant dominant follicle produces increasing amounts of estradiol which, after reaching a threshold level, is responsible for eliciting the pre-ovulatory LH surge followed by ovulation (Adams et al., 1992a; Adams, 1999).

The bovine model (Pierson and Ginther, 1984; Pierson and Ginther, 1987;Adams and Pierson, 1995) was the foundation for the discovery of follicular wave development in women (Baerwald et al., 2003a; b). As with women, the majority of interovulatory intervals in cattle are composed of either two or three waves of follicular development (Ginther et al., 1989a; Adams, 1999; Baerwald et al., 2003a; b). Furthermore, mechanisms of follicular wave emergence, selection of a dominant follicle, and ovulation in women were fundamentally similar to the same endpoints during ovarian cycles in cattle (Adams and Pierson, 1995; Baerwald et al., 2003a). The methods to control follicular wave emergence (i.e., ovarian synchronization) as well as assisted reproductive technologies (i.e., in vitro fertilization, embryo transfer) have been well developed in cattle (Bo et al., 1995; Adams, 1999; Ginther et al., 2001; Mapletoft et al., 2002). Oocytes and embryos can be easily obtained non-surgically, in a natural reproductive

cycle or after controlled ovarian stimulation for the purposes of cellular or molecular studies.

Events of prenatal ovarian development like formation of gonadal ridge (gestation Day 32), definitive ovary (gestation Day 40), initiation of meiosis (gestation Day 70-80), and primordial follicle assembly (gestation Day 150 onwards) are well-characterized in cattle and are contemporaneous to those of women (Erickson, 1966). In both species, the numbers of primordial follicles at birth are about 5% to 20 % of peak number of germ cells present in the prenatal ovary (Baker, 1963; Erickson, 1966).

By design, we used 13-14 year old cows (n = 10) and their 1-4 year old daughters (n = 10) that were born and maintained on the same farm throughout their life span. The design allowed us to minimize the effects of environmental and genetic variations, and the complicating issues of specific reproductive pathology. Cattle in the herd from which these cows were taken are selected for fertility; i.e., cows that fail to produce a calf are systematically culled. Hence, the old cows used in this study represent the most fertile of the herd and indeed had a calf in the spring preceding the start of the project. In a previous study, the mean life expectancy in cattle was 19 years and 55% of the herd was infertile by 13 years of age (Erickson et al., 1976). In another study (Bryner et al., 1990), the serum concentrations of FSH during days 6 to 12 of the estrous cycle, and pre-ovulatory estradiol appeared to be elevated in 13 year old cows. Thus we expected changes in ovarian function of 13-14 year old cows used in this study to be analogous to women approaching menopause.

CHAPTER 2

OBJECTIVES AND HYPOTHESES

The overall objective is to develop and validate a bovine model for the study of oocyte-associated infertility in women of advanced age.

2.1 Specific Objectives

To determine the effect of age on:

1. Follicular, luteal and endocrine functions.
2. The response of the hypothalamo-pituitary-ovarian axis to exogenous steroid treatments for synchronization of follicular wave emergence and ovulation.
3. The number of small (2-5 mm) antral follicles recruited into follicular waves.
4. The superstimulatory response to exogenous gonadotropin treatment.
5. The developmental competence of oocytes.
6. The meiotic maturation of oocytes.
7. Abnormalities of oocyte chromosome numbers.

2.2 Specific Hypotheses

Aging in cattle is associated with:

1. Elevated circulating concentrations of FSH and reduced concentrations of steroid hormones.
2. Increased numbers of ovarian follicles recruited into a follicular wave as a result of elevated circulating concentrations of FSH.
3. Decreased synchrony in FSH suppression after estradiol and progesterone treatment, with a subsequent decrease in synchrony of the FSH surge and follicular wave emergence.
4. Delayed LH surge and ovulation in response to an exogenous preovulatory estradiol treatment.
5. Reduced follicular and ovulatory response to exogenous gonadotropins.
6. Reduced developmental competence of oocytes.
7. Compromised oocyte meiotic maturation.
8. Higher incidence of abnormalities in oocyte chromosome numbers.

CHAPTER 3

FOLLICULAR, LUTEAL AND ENDOCRINE CHARACTERISTICS

Pritpal S. Malhi, Gregg P. Adams and Jaswant Singh

3.1 Introduction

Decreased fertility with maternal aging has been well documented in humans and animals (Erickson et al., 1976; Klein and Sauer, 2001). Infertility in women over 35 years of age has been associated with a dwindling ovarian follicular pool, altered hormone secretions, decreased conception rate, meiotic abnormalities and increased gestational attrition (Gosden and Faddy, 1994; Klein et al., 1996a; Soules et al., 2001; Burger et al., 2002; Kuliev et al., 2003; Santoro et al., 2003). Circulating concentrations of FSH in women increased in late reproductive stages (Klein et al., 1996a; Soules et al., 2001; Santoro et al., 2003), and the increase has been attributed to reduced inhibin B secretion (Klein et al., 1996a; te Velde et al., 1998; Soules et al., 2001; Burger et al., 2002; Santoro et al., 2003; Klein et al., 2004). Steroid hormone concentrations in older women did not show a consistent pattern but eventually decreased with advanced age (Klein et al., 1996a; te Velde et al., 1998; Soules et al., 2001). Reproductive aging in women has also been associated with a shortened follicular phase and thus, a shortened menstrual cycle (Klein et al., 1996a; Klein et al., 2002). The exact cause-and-effect relationships between endocrine and follicular changes and their impact on fertility remain unclear. To obviate the ethical and practical constraints that limit observational and interventional studies in humans, an animal model is required to study age-related

infertility in women. To date, however, a well characterized animal model for reproductive aging in women has not been established.

Ovarian follicular wave development in cattle has been characterized over the last two decades (Pierson and Ginther, 1984; Pierson and Ginther, 1987;Ginther et al., 1989a) and the bovine model (Adams and Pierson, 1995) was the foundation for the recent discovery of follicular wave development in women (Baerwald et al., 2003a; b). A follicular wave has been defined as the synchronous growth of a group of follicles stimulated by a surge of FSH (Adams et al., 1992b; Baerwald et al., 2003a). One follicle in each wave is selected to become dominant while others (subordinates) regress (Adams, 1993; Ginther et al., 1996; Adams, 1999; Ginther et al., 2000; Baerwald et al., 2003a). There are two or three waves of follicular development in the majority of bovine estrous cycles (Ginther et al., 1989a; Adams, 1999), and recent data suggest that the majority of menstrual cycles in women are also composed of two or three follicular waves (Baerwald et al., 2003a). Dominant follicles of waves occurring during the luteal phase are anovulatory as a result of suppression of circulating LH by progesterone (Adams, 1999). Although not critically tested, results of recent studies in women (Baerwald et al., 2003a; b) are also consistent with the notion that progesterone secretion during the luteal phase inhibits LH release, and is responsible for the ultimate regression of the dominant follicle of anovulatory waves. Demise of the previous dominant follicle, in turn, permits the emergence of a new follicular wave, and the pattern repeats itself (Adams et al., 1992a; Adams, 1999). During luteolysis, decreasing concentrations of progesterone relieves suppression of LH release from the pituitary, and LH pulse-frequency increases. In response, the extant dominant follicle produces increasing amounts of estradiol which,

after reaching a threshold level, is responsible for eliciting the pre-ovulatory LH surge followed by ovulation (Adams et al., 1992a; Adams, 1999). Follicular wave emergence in women, the number of waves during the menstrual cycle, dominant follicle selection, and ovulation of a single follicle were fundamentally similar to ovarian patterns in cattle (Adams and Pierson, 1995; Baerwald et al., 2003a), and provide justification for proposing the use of a bovine model to study ovarian function in women (Adams and Pierson, 1995).

In one study, the mean life expectancy in cattle was 19 years and 55% of the herd was infertile by 13 years of age (Erickson et al., 1976). Data from another study (Bryner et al., 1990) indicates that serum concentrations of FSH during days 6-12 of the estrous cycle, and preovulatory estradiol appeared to be elevated in 13 year-old cows (Bryner et al., 1990) but this study was limited to hormonal profiles and data were not studied in relation to follicular development. Thus we expected changes in endocrine, follicular and luteal function of 13-14 year old cows used in this study to be analogous to women approaching menopause.

The objectives of this study were to characterize age related temporal changes in follicular, luteal and endocrine functions, and to investigate the validity of old cows as a physiological model for human reproductive aging. We tested the hypotheses that aging in cattle is associated with 1) elevated concentrations of gonadotropins and reduced concentrations of steroid hormones in systemic circulation as reported in aging women, and 2) increased recruitment of ovarian follicles during wave emergence as a result of elevated circulating concentrations of FSH.

3.2 Materials and Methods

The experiment was conducted on non-lactating, non-pregnant, crossbred Hereford cows (13-14 year old, n = 10) and their daughters (1-4 year old, n = 10) during the months of June and July. The cows were at least 45 days post-partum and had a corpus luteum as determined by initial ovarian ultrasonography. Animals were maintained in single outdoor corral at Goodale Research Farm, University of Saskatchewan. The experimental protocol was approved by the University Committee on Animal Care and Supply under the guidelines of the Canadian Council on Animal Care.

3.2.1 Ovulation Synchronization

Ovulation was synchronized among cows using an estradiol and progesterone treatment protocol (Martinez et al., 2000; Bo et al., 2002). Estradiol-17β (5 mg; Catalog # E8875, Sigma Chemical Company, St. Louis, Missouri, USA) and progesterone (100 mg; Catalog # P0130, Sigma Chemical Company, St. Louis, Missouri, USA) were dissolved in benzyl alcohol (0.4 mL; Catalog # B27354, BDH Inc., Toronto, Ontario, Canada), mixed with canola oil (2 mL; No name®, Montreal, Quebec, Canada) and given intramuscularly. An intravaginal progesterone releasing device containing 1.9 g of progesterone (CIDR-B®, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) was inserted at the time of steroid treatment and maintained in place for 7 days. On the day of CIDR removal, a luteolytic dose of prostaglandin analog was given intramuscularly (Cloprostenol 500 μg; Estrumate®, Schering Canada Inc., Pointe-Claire, Quebec, Canada). To synchronize the preovulatory LH surge and ovulation, 1 mg of estradiol-17β in canola oil was given intramuscularly 24 h after prostaglandin treatment (Bo et al., 2002).

3.2.2 Ovarian Ultrasonography

Transrectal ovarian ultrasonography was performed daily by the same operator using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900, Instruments for Science and Medicine, Vancouver, British Columbia, Canada). Ultrasound examinations were initiated on the day of CIDR insertion to record follicular and luteal (CL) development for one complete inter-ovulatory interval (IOI: defined as the period between two consecutive ovulations). Ovarian sketches were made during each examination to record the size and relative location of the CL and follicles ≥4 mm in diameter. Follicle diameter was recorded as the average of antral size measured in two perpendicular planes (Pierson and Ginther, 1987; Baerwald et al., 2003a). The diameter of the CL was recorded similarly. The total numbers of follicles ≥2 mm were also counted in both ovaries.

3.2.3 Plasma Sampling and Hormone Assays

Blood samples from the jugular vein were obtained every 12 h (6 am and 6 pm) in 10 mL heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey, USA) and centrifuged at 1500 g for 15 minutes. The plasma was harvested and stored at -20°C. To compare characteristics of LH pulse frequency and amplitude during expected high and low progesterone phase, serial blood samples were collected every 15 minutes for 8 h from a jugular catheter (5 mL; n = 6 cows/age group) on day 8 and 18 of the IOI (day 0 = ovulation). A jugular catheter (inner and outer diameters 1.0 and 1.5 mm, respectively) was fixed in place one day before frequent blood sampling to minimize the effects of handling stress on plasma gonadotropin concentrations.

Plasma FSH concentrations were measured by radioimmunoassay using NIAMDD-anti-ovine FSH-1 primary antibody and expressed as USDA bovine FSH-I-l

units (Evans et al., 1994; Honaramooz et al., 1998). The range of the standard curve was 0.13 to 16 ng/mL with a minimum detection limit of 0.13 ng/mL (zero ligand vs. 0.13 ng/mL, unpaired t-test, $P < 0.05$) (Chard, 1990). The intra- and inter-assay coefficients of variation were 6% and 10% for low reference samples (mean, 1.68 ng/mL) and 13% and 8% for high reference samples (mean, 3.82 ng/mL), respectively. Plasma samples from mother-daughter pairs were analyzed in the same assay to distribute inter-assay variation equally between groups.

LH concentrations were expressed as NIDDK-bLH4 units (Evans et al., 1994; Honaramooz et al., 1998). The range of the standard curve was 0.06 to 8 ng/mL with a minimum detection limit of 0.06 ng/mL. The intra- and inter-assay coefficients of variation for LH were 10% and 5% for low reference samples (mean, 0.37 ng/mL) and 5% and 4% for high reference samples (mean, 0.97 ng/mL), respectively. The PC-Pulsar program (J. Gitzen and V. Ramirez, University of Illinois, Illinois, USA) was used to characterize LH pulsatility in serial plasma samples. Pulses were identified using standard deviation criteria of height and duration (Honaramooz et al., 1998). LH pulse frequency, pulse amplitude, means, and basal concentrations were also calculated (Honaramooz et al., 1998).

Plasma progesterone concentrations were determined using a solid phase radioimmunoassay (Kastelic et al., 1999) (Catalog # TKPG5; Coat-A-Count, Diagnostics Products Corporation, Los Angeles, USA) with a minimum detection limit of 0.1 ng/mL. The intra-assay coefficients of variation were 3% (low reference), 3% (medium reference) and 4% (high reference). The inter-assay coefficients of variation were 7%

(low reference, mean 1.69 ng/mL), 7% (medium reference, mean 2.62 ng/mL) and 1% (high reference, 11.66 ng/mL).

Plasma estradiol concentrations were determined using a commercial double-antibody radioimmunoassay kit (Catalog # KE2D5; Diagnostics Products Corporation, Los Angeles, USA) with a minimum detection limit of 1 pg/mL. Estradiol standards were made in charcoal-stripped bovine serum with a range of 1 to 200 pg/mL. The intra-assay coefficients of variation for estradiol were 8% (low reference), 9% (medium reference) and 6% (high reference). The inter-assay coefficients of variation were 5% (low reference, mean 10.51 pg/mL), 6% (medium reference, mean 16.28 pg/mL) and 10% (high reference, mean 34.89 ng/mL). Estradiol data were analyzed only for the ovulatory wave to avoid confounding by estradiol 17β treatment given for ovulation synchronization.

To estimate the level of stress due to frequent blood sampling, plasma cortisol concentrations were measured in frequent plasma samples (day 8 and 18 of the IOI; 0, 2, 4, 6, 8 h samples) by competitive immunoassay (Catalog # LKCO5; Immulite, Diagnostic Products Corporation, Los Angeles, USA) (Etson et al., 2004). Cortisol concentrations were compared with samples from other animals not used for frequent blood sampling (day 8 and 18 of the IOI; 0 and 8 h plasma sample). The range of the standard curve was 10 to 500 ng/mL with minimum detection limit of 2 ng/mL. The intra-assay coefficient of variation for cortisol was 7% (low reference, mean 48 ng/mL), 7% (medium reference, mean 122 ng/mL) and 6% (high reference, mean 363 ng/mL).

3.2.4 Data Analysis

Dominant and first subordinate follicles of each wave were identified by retrospective analysis of ovarian sketches. The dominant follicle was defined as the largest follicle of the wave, first identified at 4-5 mm in diameter. The first subordinate follicle was defined as the second largest follicle originating from same cohort of follicles (Ginther et al., 1989a; Adams et al., 1994a; Jaiswal et al., 2004). The day of follicular wave emergence was defined as the day when the dominant follicle was first detected at 4-5 mm of diameter (Ginther et al., 1989a; Adams et al., 1994a; Baerwald et al., 2003a; Jaiswal et al., 2004). The number of waves during the IOI was identified for each cow. The proportion of cows with two- or three-wave IOI, and the proportion of mother-daughter pairs with the same or different wave patterns were analyzed by Fisher's exact test. Inter-wave intervals (IWI), defined as the period between emergences of two successive waves were calculated. Single point numerical data (e.g., day of wave emergence, IOI, IWI, ovulatory diameter) were compared between old and young cows by Student's t-test.

To characterize day-to-day changes in follicle numbers, follicles were categorized according to diameter (2-3 mm, 4-5 mm and 6-8 mm). Data were centralized to wave emergence and analyzed by analysis of variance for repeated measures using the mixed procedure of the Statistical Analysis System (SAS; version 8.2 for MS Windows, SAS Institute Inc, North Carolina, USA) to determine the effects of age (old cows vs. daughters) and day of wave (Littell et al., 2000; Jaiswal et al., 2004). Similarly, the diameters of the dominant and first subordinate follicles of each wave, and gonadotropin data (FSH, LH) were centralized to wave emergence to determine the effects of age (old cows vs. daughters) and day of the IOI by analysis of variance for repeated measures. To

determine temporal association between FSH peaks and wave emergence, FSH data were also analyzed by centralization to the peak of FSH (defined as highest value of FSH detected before wave emergence). Plasma LH concentrations in frequent blood samples were analyzed to compare LH pulse frequency, pulse amplitude, mean and basal concentrations between groups (old cows vs. daughters) on day 8 vs. day 18 of the IOI (day 0 = ovulation). Cortisol data were analyzed to determine the effect of age, day of IOI (day 8 vs. day 18) and sampling (frequently sampled animals vs. others not used for frequent plasma sampling).

Corpus luteum (CL) diameters and plasma progesterone concentrations were compared between groups from 0 to 15 days after the first ovulation of the IOI and from 0 to –5 days from the second ovulation by analysis of variance for repeated measures. Plasma estradiol values were centralized to the second ovulation (day 0 to day –7) and analyzed by analysis of variance for repeated measures to determine the effects of age during the development of the ovulatory follicle.

3.3 Results

3.3.1 Interovulatory Interval and Wave Characteristics

The ovulation synchronization procedure resulted in tight synchrony among cows. Ovulation was detected between 48 to 72 h after prostaglandin treatment in 19 of 20 cows (95%). The remaining cow ovulated 192 h after prostaglandin treatment and had a short interovulatory interval (9 days); therefore, data from this daughter were excluded from analyses. The proportion of cows with two follicular waves during the IOI was similar (P = 0.6) between old cows (6/10; 60%) and their daughters (7/9; 78%). The remainder had three waves of follicular development during the IOI. The difference in the proportion of

mother-daughter pairs with the same vs. different wave patterns (6/9 vs. 3/9) was not significant (P = 0.35).

The duration of the IOI for 2-wave and 3-wave patterns was 20 and 23 days, respectively (P = 0.003). The mean days of wave emergence were 0.5 ± 0.1 and 10.5 ± 0.4 for 2-wave IOI, and 0.5 ± 0.2, 9.0 ± 0.5 and 15.8 ± 0.7 for 3-wave IOI. For constructing day-to-day profiles (Fig. 3.1, 3.3, 3.6), the mean days of wave emergence were rounded off to day 0 and 10 for 2-wave IOI and 0, 9 and 16 for 3-wave IOI. The day of emergence of wave 1 was similar (P = 0.88) between 2-wave (0.5 ± 0.1, n =13) and 3-wave IOI (0.5 ± 0.2, n = 6), but wave 1 emerged later (P = 0.04) in old cows than in their daughters (0.7 ± 0.2, n = 10 vs. 0.2 ± 0.2, n = 9 respectively; data combined from 2- and 3-wave IOI's). Remaining characteristics of follicular waves during the interovulatory interval in old cows and their daughters are summarized in Table 3.1. There was no difference between age groups in the IOI, IWI, or the day of wave emergence.

3.3.2 Follicle Numbers

Changes in the number of follicles in different size categories (2-3 mm, 4-5 mm and 6-8 mm) are illustrated in Figure 3.1. No difference between age groups was detected in the number of 2-3 mm follicles per wave (Fig. 3.1A), but fewer (P = 0.01) 4-5 mm follicles were detected in wave 1 of old cows with 2-wave IOI than in that of their daughters (Fig. 3.1B). For all follicular waves combined, fewer (P = 0.04) 4-5 mm follicles were detected during the period encompassing wave emergence in old cows compared to their daughters (old, n = 24 waves vs. daughters, n = 20 waves; Fig. 3.2A). This was also reflected in a lower peak number of 6-8 mm follicles in old cows (specific day comparison P = 0.02; Fig. 3.1C).

Table 3.1 Comparison (mean ± SEM) between old cows and their daughters in follicular wave characteristics (expressed in days) during one interovulatory interval.

End Point (Days)	Old Cows	Daughters	*P*-Value
2-wave interovulatory intervals			
	n = 6	n = 7	
IOI[a]	20.0 ± 0.6	20.6 ± 0.5	0.50
IWI[b]			
Wave 1	9.8 ± 0.7	10.3 ± 0.5	0.60
Ovulatory Wave	9.5 ± 1.1	10.0 ± 0.7	0.69
Wave emergence[c]			
Wave 1	0.7 ± 0.2	0.3 ± 0.2	0.20
Ovulatory Wave	10.5 ± 0.7	10.4 ± 0.4	0.92
3-wave interovulatory intervals			
	n = 4	n = 2	
IOI[a]	22.8 ± 0.5	23.5 ± 2.5	0.68
IWI[b]			
Wave 1	8.0 ± 0.7	9.5 ± 1.5	0.35
Wave 2	6.5 ± 0.3	7.5 ± 0.5	0.18
Ovulatory Wave	7.5 ± 0.3	6.5 ± 1.5	0.38
Wave emergence[c]			
Wave 1	0.8 ± 0.3	0.0 ± 0.0	0.12
Wave 2	8.8 ± 0.5	9.5 ± 1.5	0.56
Ovulatory Wave	15.3 ± 0.8	17.0 ± 1.0	0.25

[a] Interovulatory interval; [b] Interwave interval; [c] Day 0 = ovulation

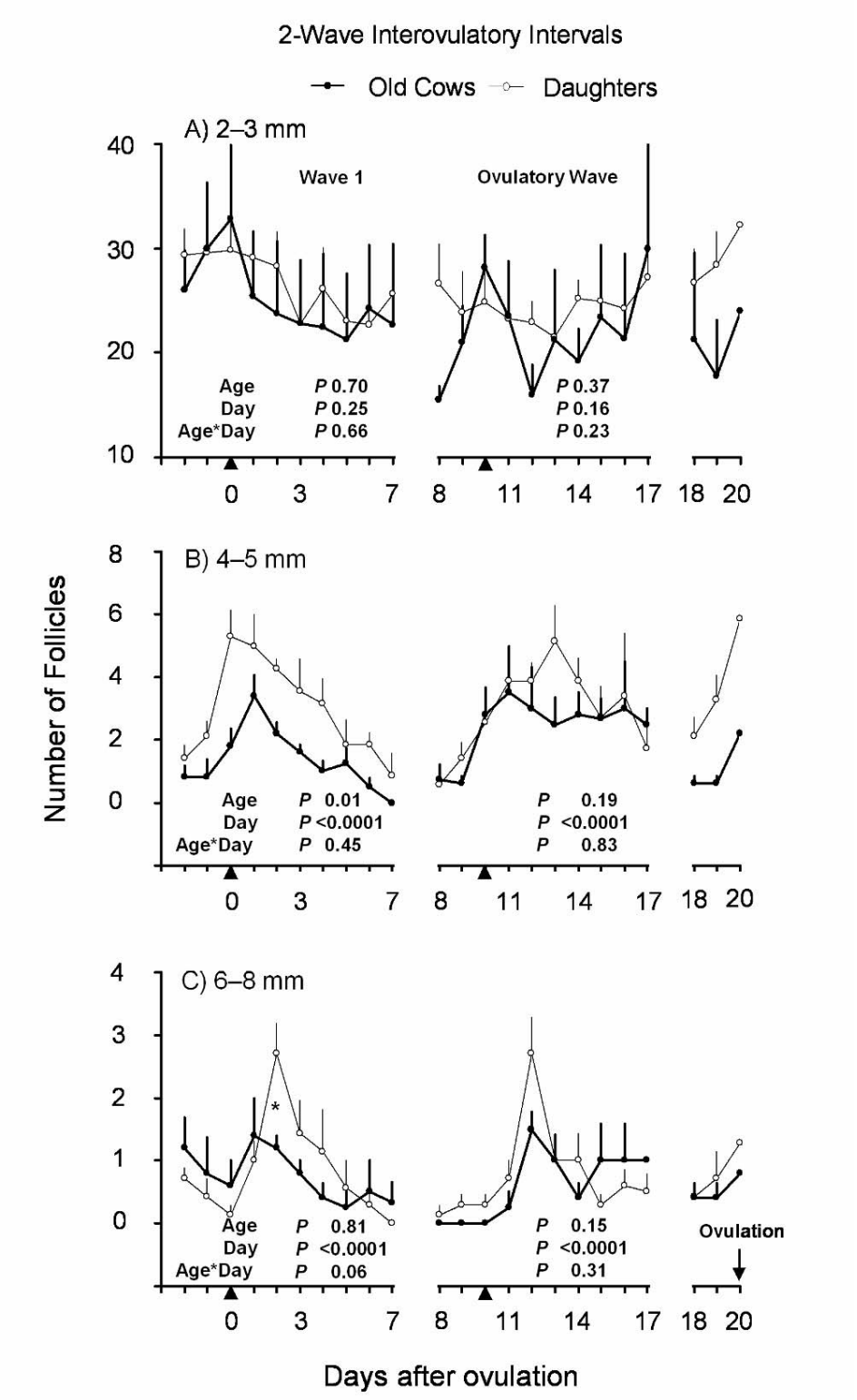

Figure 3.1 Number of follicles 2-3 mm (A), 4-5 mm (B) and 6-8 mm (C) in diameter (mean + SEM) in old cows (n = 6) and their daughters (n = 7) in 2-wave interovulatory intervals. Data for each wave were analyzed relative to the day of wave emergence (▲). *Values between groups differ (P ≤ 0.05).

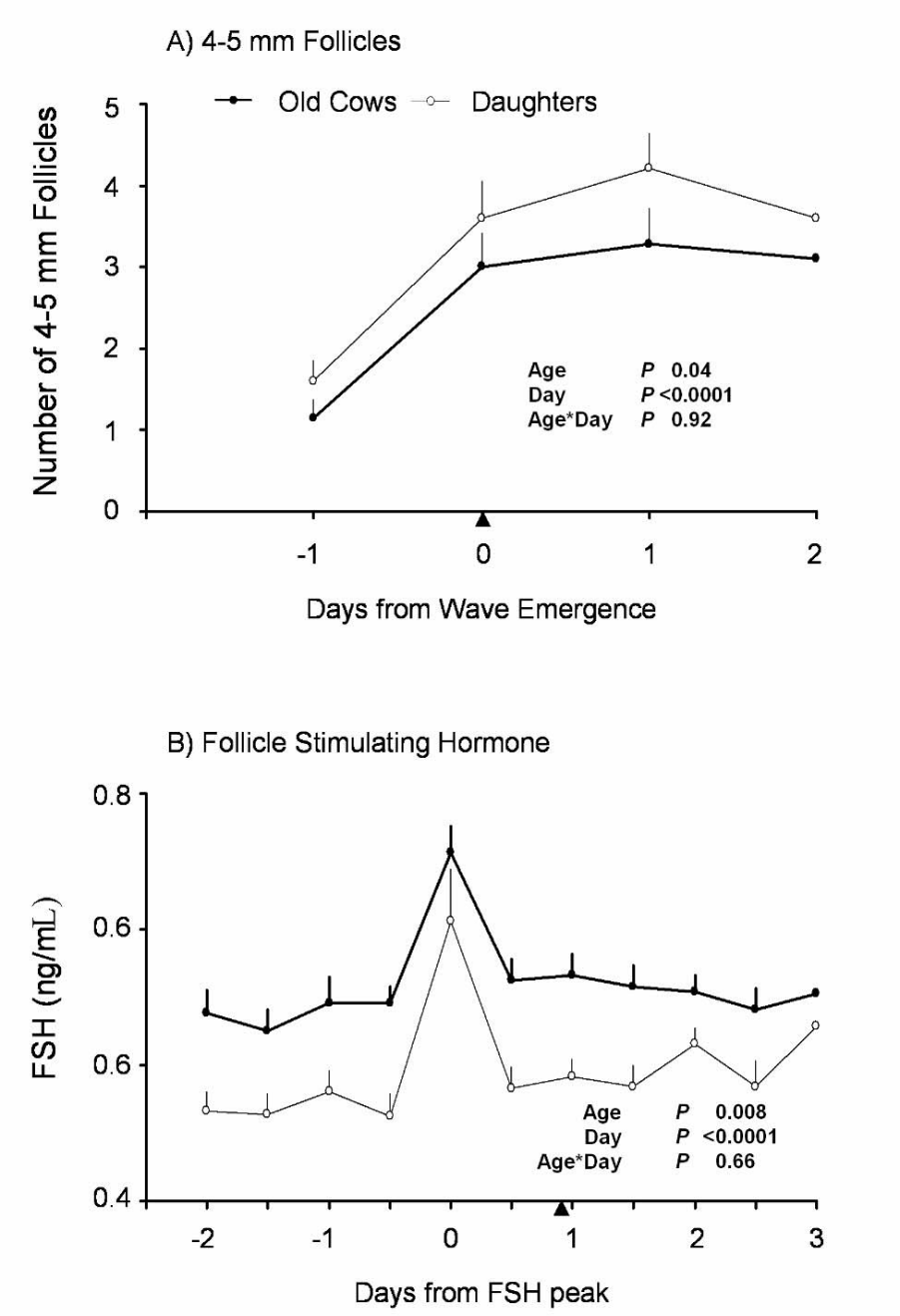

Figure 3.2 A) Changes in the number of 4-5mm follicles (mean + SEM) around the time of wave emergence (▲; all waves combined) in old cows (n = 24 waves) and their daughters (n = 20 waves). B) FSH concentrations (mean + SEM) in old cows (n = 24 waves) and their daughters (n = 20 waves) relative to the FSH peak (day 0). The FSH peak preceded wave emergence (▲) by 21 hours in old cows and 17 hours in their daughters (P = 0.4).

3.3.3 Follicle and Corpus Luteum Diameters

The diameter profiles of dominant and first subordinate follicles of anovulatory and ovulatory waves are illustrated in Figure 3.3. The diameter profile of the ovulatory follicle of old cows with 2-wave IOI was smaller than that of their daughters (P = 0.04; Fig. 3.3) whereas no age related effect was detected in cows with 3-wave IOI (P = 0.83). When data from 2- and 3-wave IOI were combined, the mean diameter of the dominant follicle on the day before ovulation was smaller in old cows than in their daughters (12.3 ± 0.5, n = 10 vs. 13.9 ± 0.5, n = 9; P = 0.04). As expected, the diameter of the dominant follicle on the day before ovulation tended to be smaller in 3-wave IOI than in 2-wave IOI (11.9 ± 0.3, n = 6 vs. 13.5 ± 0.5, n = 13; P = 0.06). There was no effect of age on diameter profiles of the first subordinate follicles in either 2-wave (P = 0.63) or 3-wave IOI (P = 0.59). In old cows with 2-wave IOI, the CL profile (day 0 to 15) tended to be smaller than in their daughters (P = 0.09, Fig. 3.4A), while no differences in CL diameter were detected of 3-wave IOI (P = 0.69, Fig. 3.5A).

3.3.4 Gonadotropins and Cortisol

Characteristics of plasma concentration of gonadotropins in old cows (n = 10) and their daughters (n = 9) are summarized in Table 3.2. Mean FSH concentrations during the IOI (averaged over all days of IOI) was higher in old cows than in their daughters (Table 3.2; plasma samples n = 42/animal/group, P = 0.01) Concentrations of FSH were significantly higher during the first wave (2-wave IOI) and during the ovulatory wave (2- and 3-wave IOI) in old cows versus their daughters (Fig. 3.6A).

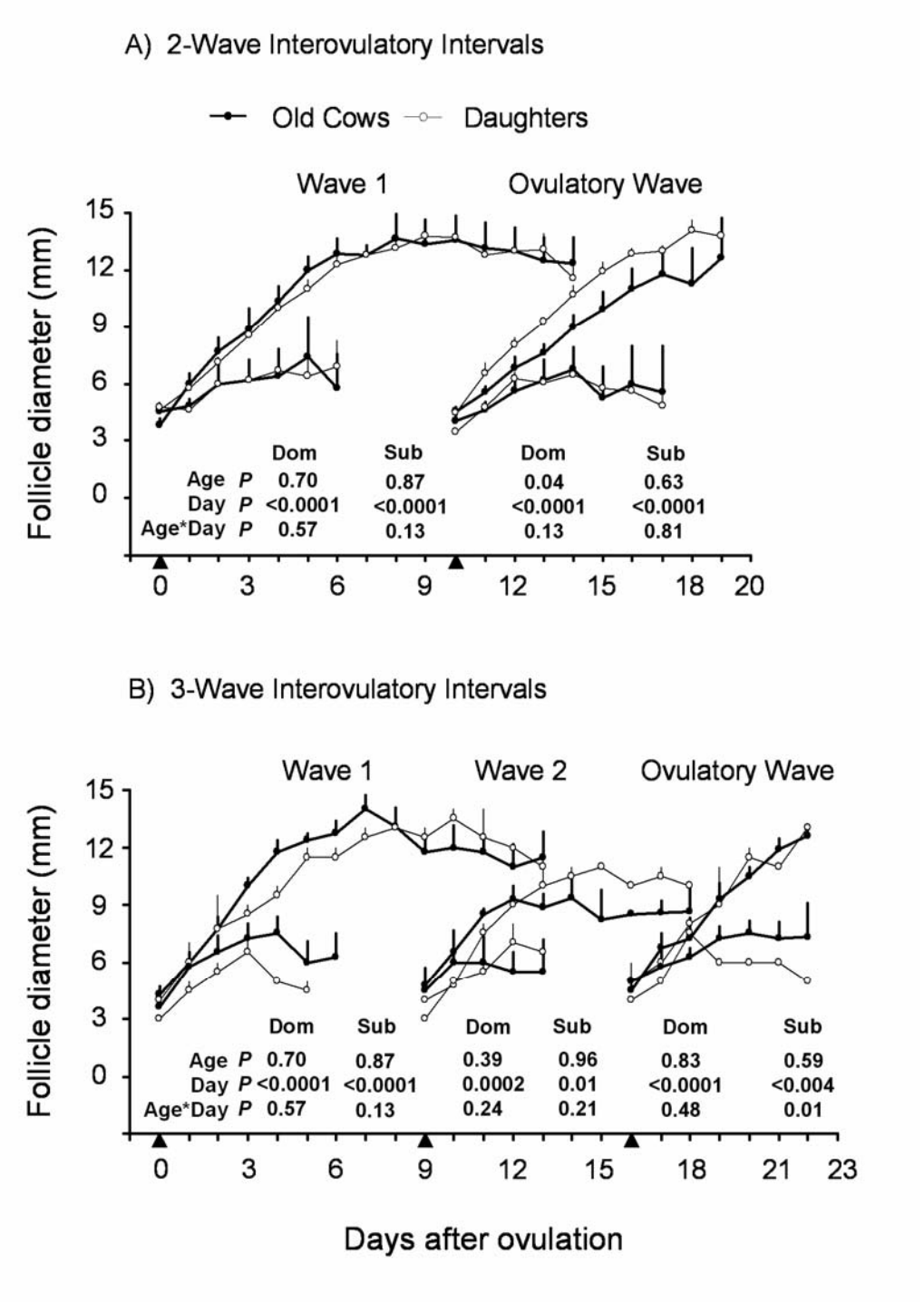

Figure 3.3 Diameter profiles (mean + SEM) of the dominant (Dom) and first subordinate (Sub) follicles in old cows (n = 6 in Fig. A and n = 4 in Fig. B) and their daughters (n = 7 in Fig. A and n = 2 in Fig. B) in 2-wave and 3-wave interovulatory intervals (▲wave emergence).

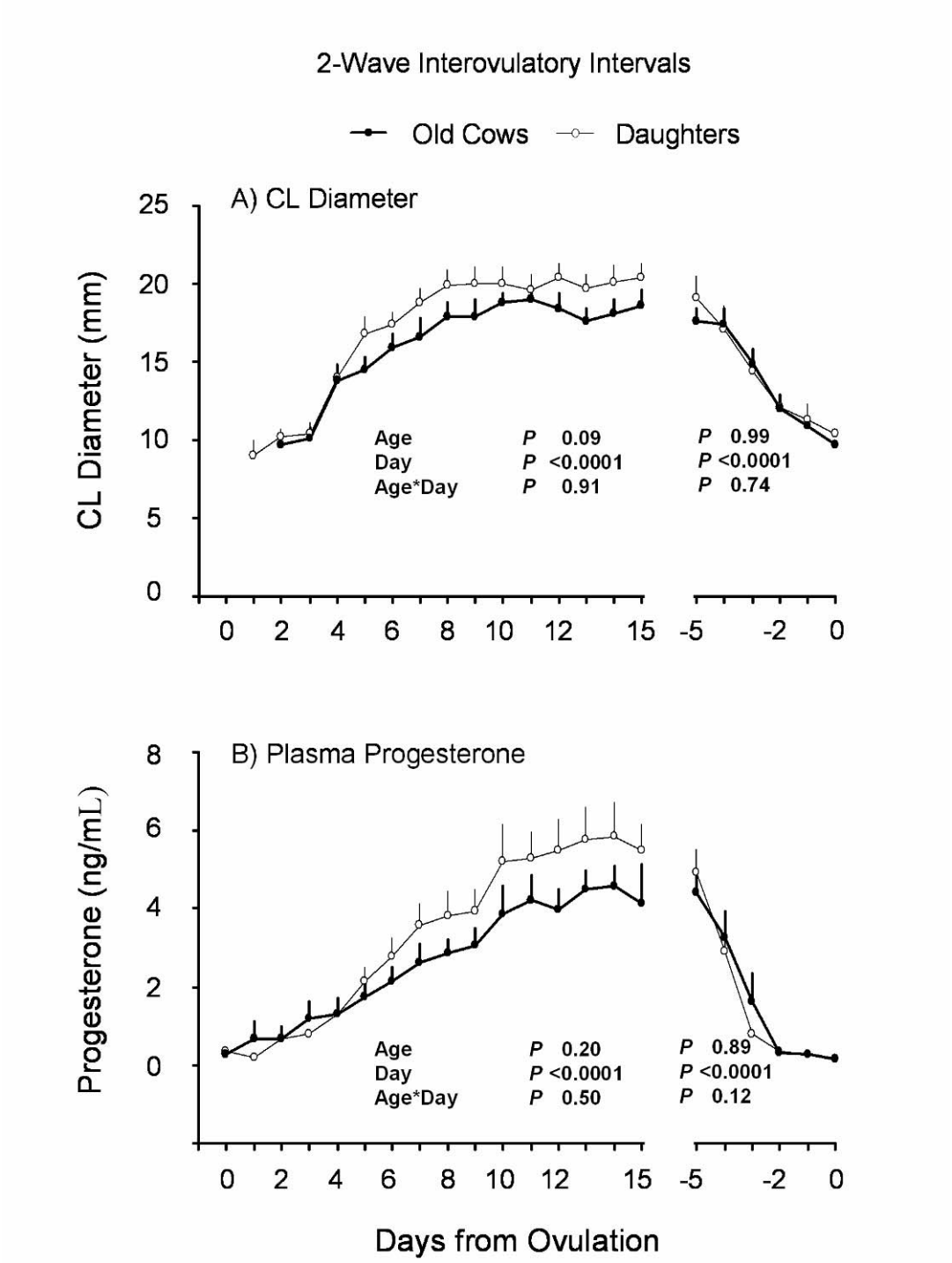

Figure 3.4 Profiles (mean + SEM) of CL diameter (A) and plasma progesterone concentration (B) in 2-wave interovulatory intervals of old cows (n = 6) and their daughters (n = 7). Data were analyzed from day 0 (ovulation) to day 15 and from day - 5 to day 0 (subsequent ovulation).

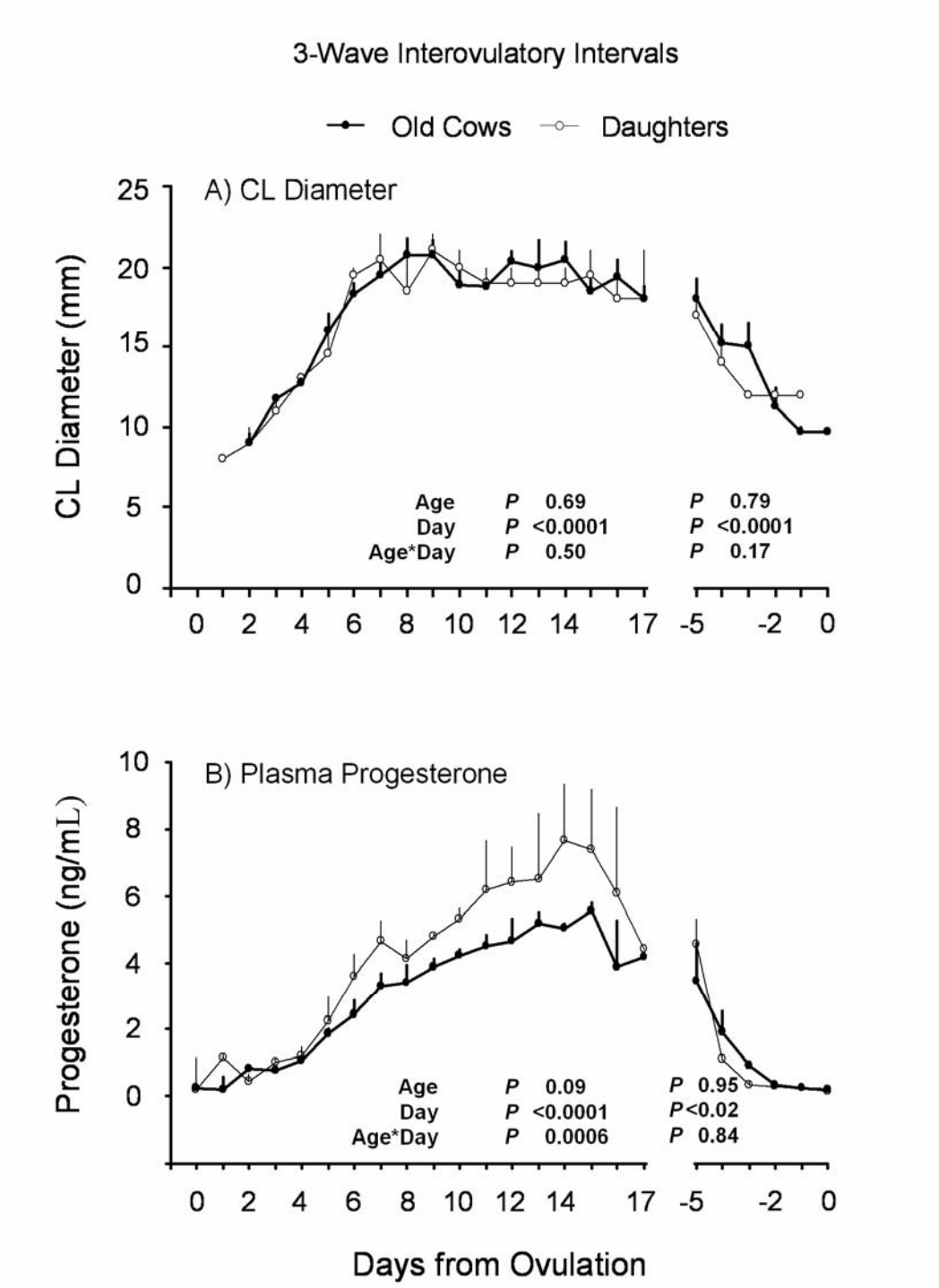

Figure 3.5 Profiles (mean + SEM) of CL diameter (A) and plasma progesterone concentration (B) in 3-wave interovulatory intervals of old cows (n = 4) and their daughters (n = 2). Data were analyzed from day 0 (ovulation) to day 17 and from day -5 to day 0 (subsequent ovulation).

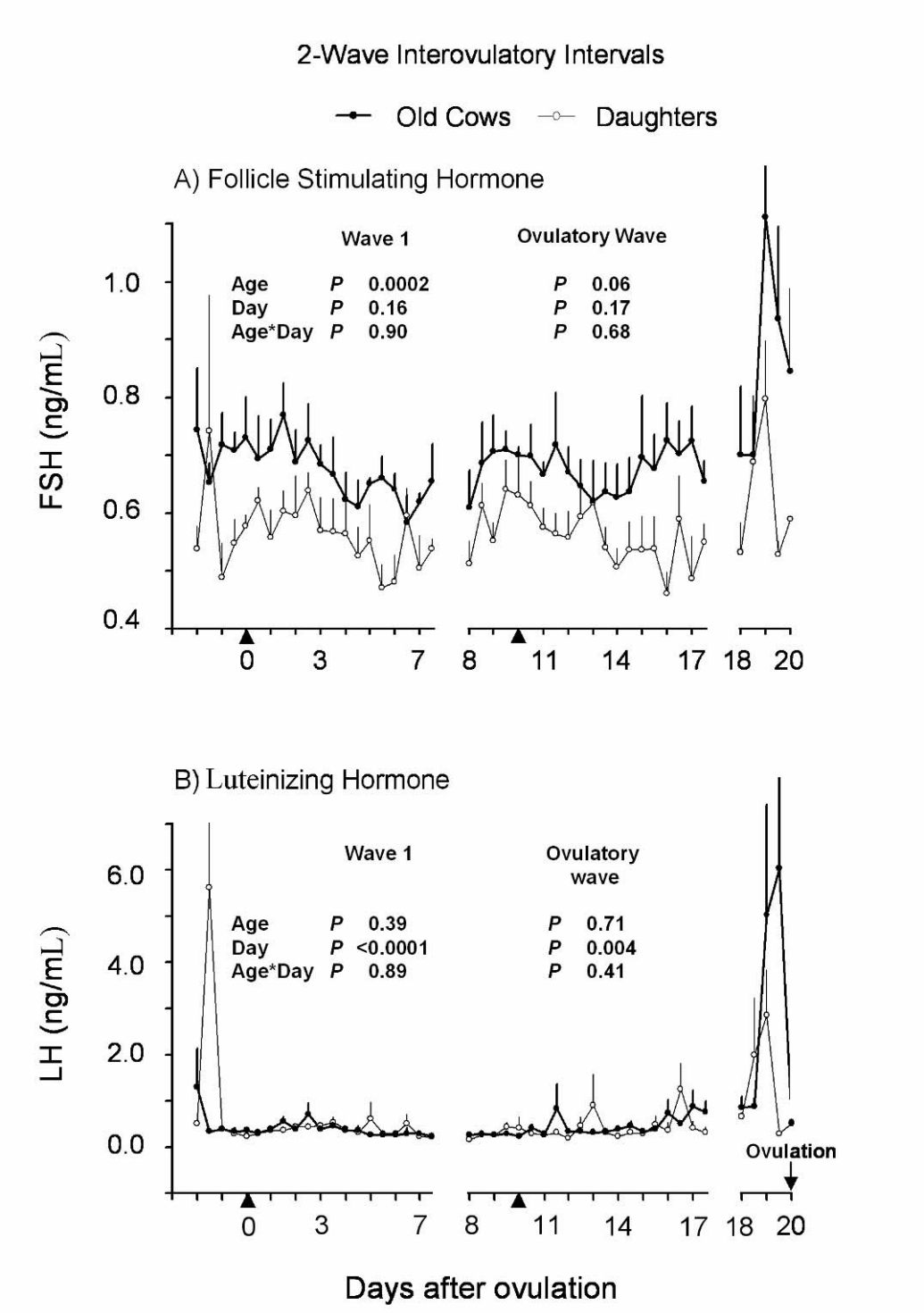

Figure 3.6 Plasma FSH (A) and LH (B) concentrations (mean + SEM) in old cows (n = 6) and their daughters (n = 7) in 2-wave interovulatory intervals. Data were analyzed relative to wave emergence (▲).

Table 3.2 Comparison (mean ± SEM) between old cows and their daughters in plasma concentrations of gonadotropins and sex steroids during one interovulatory interval.

End Point (ng/mL)	Old Cows (n = 10)	Daughters (n = 9)	*P*-Value
Mean concentration during IOI[a]			
FSH	0.70 ± 0.03	0.58 ± 0.03	0.01
LH	0.61 ± 0.11	0.55 ± 0.07	0.63
Progesterone	2.51 ± 0.21	3.18 ± 0.42	0.16
Luteal phase concentration (Day 8 to 15)			
Mean Progesterone	4.16 ± 0.34	5.31 ± 0.59	0.10
Peak progesterone concentration[b]	5.50 ± 0.42	6.97 ± 0.78	0.11
Day of peak progesterone concentration	14.00 ± 0.33	13.78 ± 0.52	0.72
Mean Estradiol[c]	1.76 ± 0.50	0.76 ± 0.40	0.14
Preovulatory concentration[d]			
Peak FSH	1.13 ± 0.42	1.09 ± 0.13	0.82
Peak LH	7.65 ± 3.06	6.31 ± 2.35	0.73
Peak Estradiol	9.78 ± 1.44	6.77 ± 1.24	0.13
Basal progesterone[e]	0.25 ± 0.04	0.26 ± 0.02	0.73
Time of preovulatory peak concentration (hours before ovulation)			
FSH	18.00 ± 4.47	26.40 ± 3.92	0.17
LH	30.00 ± 4.82	21.60 ± 3.92	0.19
Estradiol	19.20 ± 2.65	21.60 ± 2.99	0.56

[a] Interovulatory interval; [b] Highest concentration detected during IOI; [c] Estradiol expressed in pg/mL; [d] Highest concentration detected before ovulation; [e] Mean of progesterone concentrations in last three samples

A combined analysis of all waves (old, n = 24 waves vs. daughters, n = 20 waves; day -2 to + 3 from wave emergence) demonstrated higher circulating FSH concentrations in older cows (P = 0.009). When data were centralized to FSH peak (old, n = 24 waves vs. daughters, n = 20 waves; Fig. 3.2B), day (P < 0.0001) and age effects (P = 0.008) were observed, but the period between the FSH peak and wave emergence was identical in both age groups. There were no differences between young and old cows in the pre-ovulatory FSH peak concentration or time of its occurrence (Table 3.2). No differences between age groups were detected in mean plasma concentration of LH during the IOI or during the pre-ovulatory LH surge (Table 3.2, Fig. 3.6B). LH characteristics were studied in frequent plasma samples collected on day 8 and 18 of IOI (Table 3.3). As expected, there was a higher number of LH pulses during the low progesterone phase (day 18 of IOI) than during the mid-luteal phase (day 8; P = 0.003). The mean number of LH pulses, pulse amplitude, means and basal concentrations (Table 3.3) were not different between age groups. Cortisol concentrations were not different among age groups (P = 0.23) and day of IOI (P = 0.43) for cows from which frequent blood samples were taken (Fig. 3.7A-B). Cortisol concentrations were not different between cows from which frequent blood samples were taken or not (P = 0.61, Fig. 3.7C-D).

3.3.5 Progesterone and Estradiol

Progesterone concentrations in cows with 2-wave and 3-wave patterns changed over days of IOI (P < 0.0001; Fig. 3.4B, 3.5B). A tendency for lower circulating progesterone (P = 0.09) was observed in old cows with 3-wave IOI. When data were combined between 2-wave and 3-wave IOI, luteal phase progesterone concentrations (day 8 to 15) tended to be lower in old cows than in their daughters (P = 0.10; Fig. 3.8A).

Table 3.3 Comparison (mean ± SEM) of plasma LH characteristics between old cows (n = 6) and their daughters (n = 6) in serial samples obtained every 15 minutes for 8 hrs (n = 33) on day 8 and 18 (Day 0 = ovulation).

End Point	Day 8 of IOI		Day 18 of IOI		P-Value
	Old Cows	Daughters	Old Cows	Daughters	
Mean (ng/mL)	0.29 ± 0.04	0.26 ± 0.02	0.57 ± 0.21	0.36 ± 0.06	Age 0.34 Day 0.10
Basal concentration (ng/mL)	0.22 ± 0.04	0.19 ± 0.02	0.46 ± 0.15	0.25 ± 0.05	Age 0.14 Day 0.07
Number of pulses	4.00 ± 0.37	4.83 ± 0.40	7.83 ± 1.19	6.67 ± 1.61	Age 0.61 Day 0.003
Pulse amplitude (ng/mL)	0.23 ± 0.03	0.23 ± 0.06	0.27 ± 0.09	0.34 ± 0.10	Age 0.96 Day 0.60

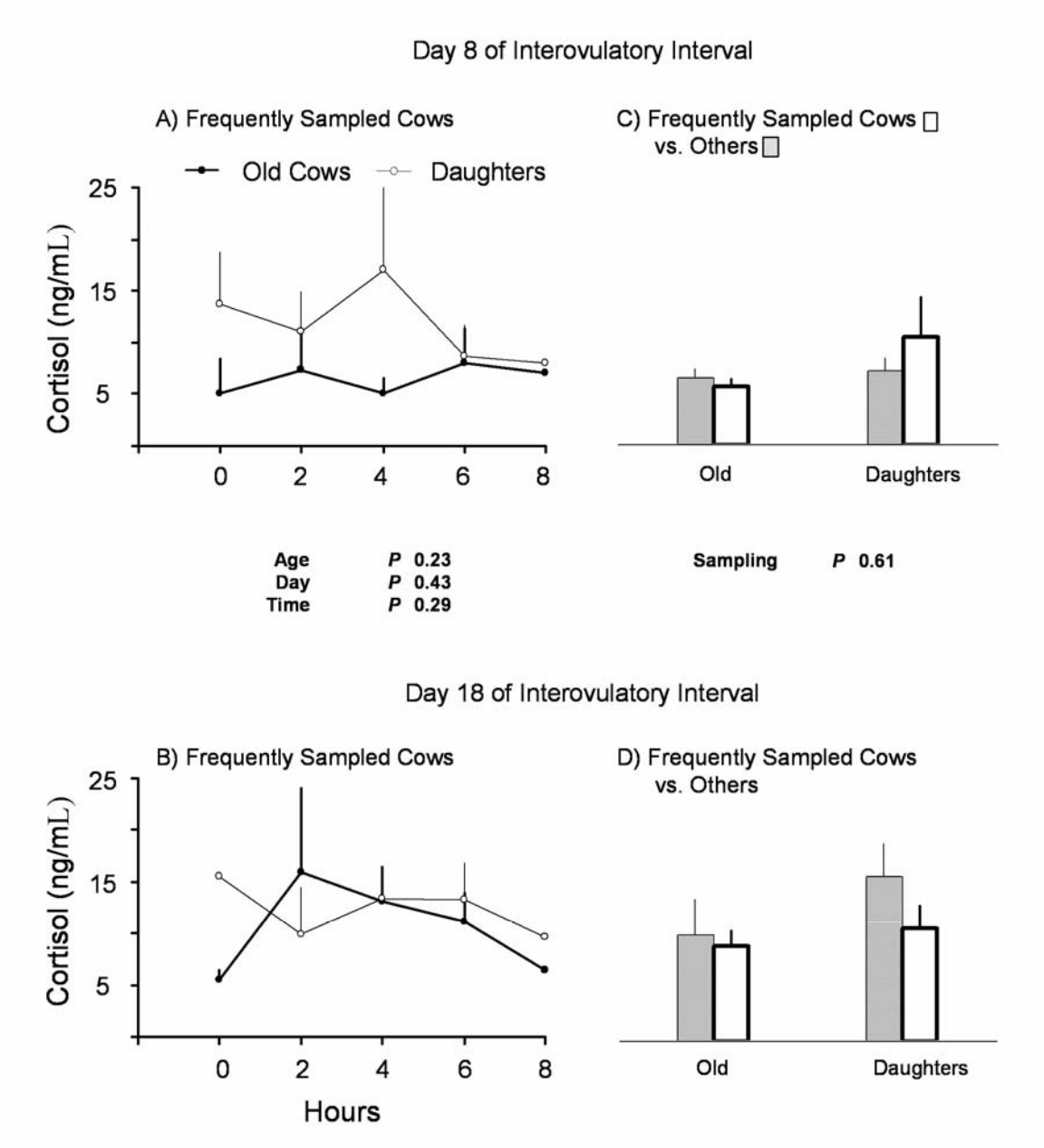

Figure 3.7 Plasma cortisol concentrations (mean + SEM) in old cows (n = 6) and their daughters (n = 6) during an 8-hour sampling period on day 8 (A) and day 18 (B; day 0 = ovulation), and a comparison of cortisol concentrations in plasma samples of cows that were frequently sampled and others not used for frequent plasma sampling (C, D).

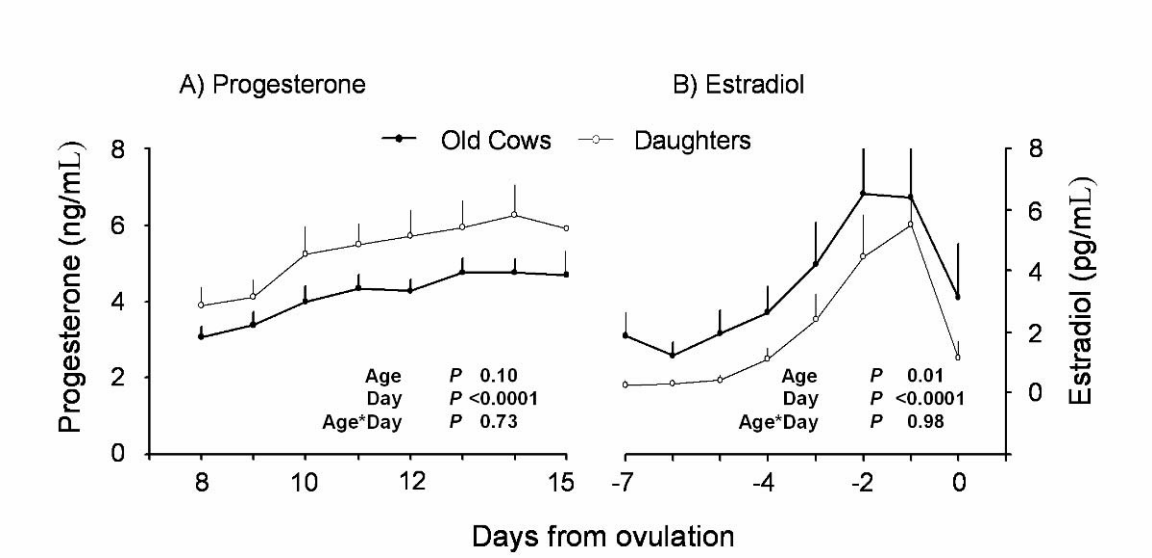

Figure 3.8 Plasma concentrations of progesterone during the luteal phase (A; day 8 to 15), and estradiol during the preovulatory phase (B; day -7 to 0) in old cows (n = 10) and their daughters (n = 9).

Mean and peak plasma progesterone concentrations during the IOI were numerically lower in old cows, but differences were not statistically significant (P = 0.16 and 0.11, respectively; Table 3.2). The profile of plasma concentrations of estradiol during the 7 days preceding ovulation was greater (P = 0.01) in old cows than in their daughters (Fig. 3.8B). There was a progressive increase in estradiol levels in both age groups (day effect, P < 0.0001). The preovulatory peak in estradiol concentrations, and the time of its occurrence were not different between age groups.

3.4 Discussion

Based on similarities in ovarian follicular dynamics and endocrine control in cattle and women (Adams and Pierson, 1995; Baerwald et al., 2003a), the bovine model has been proposed for studying reproductive events in women (Adams and Pierson, 1995). This study was designed to characterize age related changes in follicular, luteal and endocrine function in cattle approaching reproductive senescence and to validate the existing bovine model for study of reproductive events in women approaching menopause. Based on the existing human literature, we hypothesized that reproductive aging in cattle will be associated with elevated circulating concentrations of gonadotropins, reduced concentrations of steroid hormones, and accelerated follicle recruitment during wave emergence.

Ovulation was synchronized using estradiol and progesterone for logistical purposes, and to minimize the effects of environment and nutrition on follicular development and endocrinology over time. Follicular dynamics following estradiol and progesterone treatment have been well characterized (Bo et al., 1995; Adams, 1998), and follicle numbers or diameters of the three largest follicles were similar to that of spontaneous waves. Furthermore, cows synchronized with a similar protocol had

pregnancy rates comparable to untreated controls (Martinez et al., 2000; Bo et al., 2002). Therefore, we expected the interovulatory cycles after ovulation synchronization in present study to be similar to those occurring naturally. Cattle in the herd from which these cows were taken are selected for fertility; i.e., cows that fail to produce a calf are systematically culled. Hence, the old cows used in this study represent the most fertile of the herd and indeed had a calf in the spring preceding the experiment. Based on one report (Erickson et al., 1976) that 55% of the herd is infertile by 13 years of age; old cows in the present study represent the top half of the herd in terms of fertility. By design, we used 13-14 year old cows and their 1-4 year old daughters that were born and maintained on the same farm throughout their life span. The design allowed us to minimize the effects of environmental and genetic variations, and the complicating issues of specific reproductive pathology.

Similar to women in advanced reproductive age (Klein et al., 1996a; Soules et al., 2001; Burger et al., 2002; Santoro et al., 2003), we detected that mean plasma FSH concentrations during interovulatory interval as well as circulating FSH concentrations in follicular waves were consistently higher in old cows than in their daughters. A rise in circulating FSH concentrations was considered the first sign of reproductive aging in women (Soules et al., 2001) despite that the women still had regular menstrual cycles. Results of the present study extend those of a previous study done in older cattle in which elevated circulating FSH concentrations were detected during day 6 to 12 of estrous cycle (Bryner et al., 1990). Data were not analyzed relative to follicular wave emergence in the later study, but it is noteworthy that elevated FSH concentration observed in old cows in the present study followed the expected pattern associated with wave emergence; i.e.,

each wave was preceded by a surge in circulating FSH (Fig. 3.2B). An age related rise in urinary FSH-β subunit was also reported in rhesus monkeys with irregular menstrual cycles. However, unlike women, an increase in FSH was not observed in middle-aged monkeys with regular menstrual cycles (Bellino and Wise, 2003).

The follicular wave pattern was maintained in the old cows and the majority of mother-daughter pairs had the same wave pattern (6 pairs out of 9). The 2-wave pattern occurred in 60% of the estrous cycles of old cows and the 3-wave occurred in the remainder, similar to their daughters. This pattern is also consistent with that observed in previous studies in heifers (Ginther et al., 1989a; Ginther et al., 1989b; Knopf et al., 1989; Singh and Adams, 1998; Singh et al., 1998; Singh and Adams, 2000) and is consistent with the results of a recent study in normal young (mean, 28 years) women (Baerwald et al., 2003a). The hereditary, nutritional, and environmental factors affecting wave patterns are not well understood but in one study, cows fed high and low energy rations favored 2- and 3-wave patterns respectively (Murphy et al., 1991). The repeatability of wave patterns, if any, is also not understood and no conclusive hereditary inferences could be drawn from the limited number of mother-daughter pairs in this study.

The length of interovulatory and interwave intervals did not change with age in the present study, similar to an earlier study in cattle where the length of estrous cycle did not differ among age groups (Bryner et al., 1990). A tendency for shorter menstrual cycles in older women was reported and has been attributed to a short early follicular phase (interval from onset of menstruation to FSH rise) (Klein et al., 1996a; Klein et al., 2002). Authors suggested that the early FSH rise in older women resulted in early

dominant follicle selection (Klein et al., 2002); however, others (Baerwald et al., 2003a) have shown that the FSH rise is unrelated to the onset of menstruation and is associated with follicular wave recruitment rather than selection of the dominant follicle. The duration of the ovulatory wave (period from wave emergence to ovulation) was similar between old cows and their daughters in the present study. Similarly, the length of the late follicular phase in women (period from initial FSH rise to preovulatory gonadotropin surge) was not different between age groups (Klein et al., 2002).

The first wave of the estrous cycle in old cows emerged 12 h later than in their daughters, but there was no such age effect on emergence of other waves. It is interesting to note that despite the short delay in emergence of the first wave, the interval between the preovulatory gonadotropin surge and ovulation at the end of our study period, and the interval between exogenous estradiol and induced ovulation at the beginning of the study were not affected by age. This appears contrary to a study in older women in which an early FSH peak was detected after recovery from hypothalamic-pituitary-gonadal axis suppression (Klein et al., 2002).

Fewer 4-5 mm follicles were recruited into a wave in old cows even though comparable numbers of 2-3 mm follicles were available at the time of wave emergence in young and old cows. This is contrary to our hypothesis that higher FSH concentrations in old cows would result in greater follicular recruitment. This hypothesis was also based on a presumed increased rate of follicle loss during menopause transition (Gosden and Faddy, 1994) as levels of FSH rise (Klein et al., 1996a; Soules et al., 2001; Burger et al., 2002; Santoro et al., 2003), and on recent studies in cattle in which 1-3 mm follicles were found to be sensitive to FSH and develop in a wave like pattern (Jaiswal et al., 2004).

Based on our results, we speculate that increased FSH in old cows is able to stimulate small (2-3 mm) follicles from the dwindling ovarian follicular pool but may not be able to sustain their growth beyond the initial stages of wave emergence. In this study, we did not assess the rate of primordial, preantral and early antral (<2 mm) follicle loss by atresia, or their contributions to age-related follicle number decline. Reduced recruitment of 4-5 mm follicles into the follicular wave despite elevated circulating concentrations of FSH in old cows may be a result of 1) reduced numbers of granulosa cells in follicles, 2) reduced numbers of gonadotropin receptors per granulosa cell 3) impaired receptor-hormone binding, 4) reduced responsiveness of granulosa cell after receptor-hormone binding, or 5) changes in the intrinsic ovarian follicle growth factor systems. Altered receptor-hormone interactions may also be involved in the decrease in superovulatory response reported in older women (Kim, 1995) and cattle (Lerner et al., 1986). In this regard, an age-related reduction in binding of FSH to its receptors was demonstrated in FSH-R heterozygous and wild-type mice (Danilovich et al., 2002). FSH-R heterozygous mice show a rise in FSH levels with age, undergo accelerated follicle loss and reproductive aging and have a shorter reproductive life than wild type mice (Danilovich and Sairam, 2002).

The ovulatory follicle of old cows with a 2-wave pattern was smaller at the time of ovulation than that of young cows in the present study. The ovulatory follicle grew for the same number of days as in the daughters (no difference in duration of the ovulatory wave) but apparently at a slower rate (Fig. 3.3). This may also be due to reduced numbers or sensitivity of gonadotropin receptors in follicles of aging ovaries. A tendency for a smaller ovulatory diameter was observed in older women in some studies (Klein et al.,

1996a; Santoro et al., 2003), but not in others (Klein et al., 2002). It is paradoxical that although the diameter of the ovulatory follicle was smaller in old cows, ovulatory wave estradiol concentrations were higher in old cows than in their daughters. The latter is consistent with previous studies in which higher estradiol concentrations were reported during the preovulatory phase in older cattle (Bryner et al., 1990) and during the follicular phase in older women (Klein et al., 1996a).

Corpus luteum diameter tended to be smaller in old cows in the present study and may be associated with a smaller ovulatory follicle diameter. Luteal diameters correlated well with the observation that old cows in this study tended to have lower circulating concentrations of progesterone during the luteal phase, similar to the findings in an earlier study (Bryner et al., 1990). In women, progesterone concentrations also decrease during menopause transition (Soules et al., 2001). The cause-and-effect relationship between low progesterone and subsequent age-related effects on follicular dynamics (i.e., smaller ovulatory follicle, fewer 4-5 mm follicles in the first wave, and delayed emergence of the first wave) remains to be elucidated.

There was no age effect on circulating LH concentrations or LH pulse frequency, similar to previous studies in cattle (Bryner et al., 1990) and women (Klein and Sauer, 2001; Soules et al., 2001). In both age groups, LH pulse frequency was higher during the low progesterone phase (Day 18) compared with the high progesterone phase (Day 8). The level of stress (assessed by circulating cortisol concentrations) was not different between age groups or between animals from which blood samples were taken intensively (every 15 min for 8 h) or less frequently (daily).

To summarize, an increased circulating concentration of FSH was detected in old cows but there was no change in LH. Old cows had higher circulating estradiol during preovulatory phase but progesterone concentrations tended to be lower during the luteal phase. Thus, our hypothesis that aging in cattle is associated with elevated gonadotropin and reduced steroid hormone concentrations was partially supported, and these changes were consistent with those reported during early reproductive aging in women. Reduced follicular recruitment (4-5 mm) at wave emergence was observed in old cows despite comparable numbers of 2-3 mm follicles between young and old, indicating possible diminished follicular responsiveness to FSH in older cattle. Thus, our hypothesis that reproductive aging will result in increased follicular recruitment, was not supported. The ovulatory follicle grew more slowly and to a smaller maximum diameter in old versus young cows. We conclude that changes in follicular dynamics and their endocrine control in 13-14 year old cows were similar to those previously reported in women approaching menopause, and that the bovine model is suitable for the study of reproductive aging in women.

Observed differences in ovarian function between old and young cows in this study may be expected to be a conservative estimate of changes occurring during the transition to reproductive senescence and may be harbingers of age-related infertility. The bovine model may be particularly useful for addressing issues relevant to age-related infertility in women such as 1) test of the hypothesis that antral follicle count is an accurate predictor of ovarian follicle reserve, 2) study of nuclear and cytoplasmic changes in the oocyte associated with subfertility, and mechanisms associated with chromosomal aberrations, 3) identification of oocyte or granulosa cell markers of

fertility, 4) development of interventional strategies for improving ovarian stimulation and oocyte competence, and 5) elucidation of the role of telomere length and telomerase activity in aging somatic and reproductive tissues.

CHAPTER 4

OVARIAN SYNCHRONIZATION AND SUPERSTIMULATION

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4.1 Introduction

Ovarian follicles develop in waves during bovine estrous cycles (Ginther et al., 1989a; Adams, 1999). A follicular wave has been defined as the synchronous growth of a group of follicles stimulated by a surge of FSH (Adams et al., 1992b; Baerwald et al., 2003a). In cattle, there were either two or three waves of follicular development in most interovulatory intervals (Ginther et al., 1989a; Adams, 1999) and the follicular wave pattern was maintained in older cattle (Malhi et al., 2005). Little critically derived information is available on reproductive aging in cattle, however, recent work from our laboratory has demonstrated that circulating concentrations of FSH increase and the number of 4 to 5 mm follicles recruited into each wave decreases with maternal age (Malhi et al., 2005). In an early study (Erickson et al., 1976), aging was associated with a decrease in the ovarian follicular reserve and 55% of the herd was reported to be infertile by 13 year of age. In recent studies (Baerwald et al., 2003a; b), ovarian activity during the menstrual cycle of women was also composed of either two or three follicular waves; follicular wave emergence, selection of a dominant follicle, and ovulation were fundamentally similar to the same endpoints during ovarian cycles in cattle (Adams and Pierson, 1995; Baerwald et al., 2003a). Furthermore, the earliest sign of reproductive aging in women is an increase in circulating concentrations of FSH

(Klein et al., 1996a), presumably due to reduced inhibin B secretion (Klein et al., 2004) and decreased ovarian follicle reserve (Gosden and Faddy, 1994; Klein et al., 2004). Ethical and practical constraints limit direct evaluation in humans; thus, a bovine model to study ovarian function and age-associated infertility in women was proposed based on similarities in follicular and endocrine characteristics between these two species (Adams and Pierson, 1995; Malhi et al., 2005).

Estradiol and progesterone treatments have been successfully used in young cows for synchronizing follicular wave emergence and ovulation (Bo et al., 1994; Bo et al., 2002). A single treatment of estradiol and progesterone at random stages of the estrous cycle suppressed circulating FSH and thus follicular development (Bo et al., 1994). A new wave of follicular development emerged 4 days after treatment as the pituitary recovered from negative steroid feedback (Bo et al., 2002). Conversely, a smaller dose of estradiol given to cows with lower endogenous progesterone induced an LH surge 18-24 h later and resulted in ovulation of the extant dominant follicle (Bo et al., 1994).

All follicular waves in cattle and major follicular waves in women are characterized by selection of one dominant follicle which continues to grow while others (subordinates) regress (Adams et al., 1993; Adams, 1999; Ginther, 2000; Baerwald et al., 2003a). Exogenous FSH treatments initiated at the time of follicular wave emergence can rescue follicles from atresia in both species, and thus stimulate multiple follicle growth (i.e., ovarian superstimulation) (Adams et al., 1993; Nasser et al., 1993; Adams et al., 1994b). In cattle, the superstimulatory response was greater when treatment was initiated near the time of wave emergence (i.e., before irreversible atresia of subordinates) than later (Nasser et al., 1993). The antral follicle count (≥ 2 mm) at the start of

superstimulatory treatment was considered a good predictor of follicular response in cattle and women (Ng et al., 2000; Singh et al., 2004). In women (> 40 year of age) who choose assisted reproductive technologies to complete their families, a reduced follicular response has been observed after ovarian superstimulation (Kim, 1995; Dew et al., 1998).

The present study was designed to determine the effect of age on 1) the responsiveness of the hypothalamo-pituitary axis to exogenous steroid treatments for synchronization of follicular wave emergence and ovulation, and 2) the superstimulatory response to exogenous gonadotropin treatment. We tested the hypotheses that aging in cattle is associated with 1) decreased synchrony in FSH suppression after estradiol and progesterone treatment, with a subsequent decrease in synchrony of the FSH surge and wave emergence, 2) delayed LH surge and ovulation in response to exogenous preovulatory estradiol treatment, and 3) a reduced ovarian response to superstimulatory doses of exogenous FSH.

4.2 Materials and Methods

The experimental protocols (Fig. 4.1) were approved by the University Committee on Animal Care and Supply under the guidelines of the Canadian Council on Animal Care. Animals were born and raised at Goodale Research Farm, University of Saskatchewan, Saskatoon (52° North and 106° West), and were maintained in single outdoor corral. All cows were at least 45 days post-partum, and were not given any hormonal treatments after partituration. All cows were non-lactating, non-pregnant, and had an ultrasonographically detectable corpus luteum at the beginning of the study. The body condition scores of both age groups were between 2.5 and 3.5.

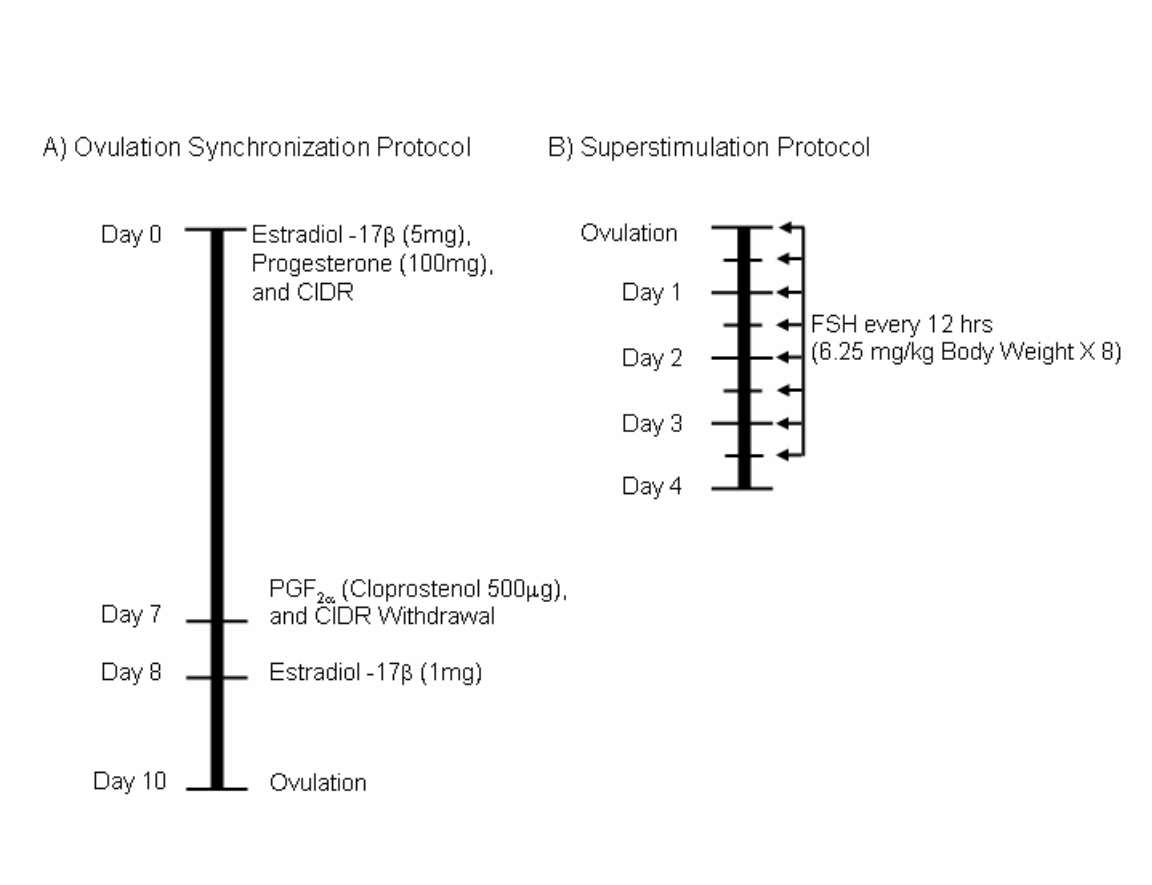

Figure 4.1 Treatment protocol for ovulation synchronization (A) and superstimulation (B)

4.2.1 Experiment 1: Ovarian Synchronization

The experiment was conducted on crossbred Hereford cows (Old cows, 13 to 14 year of age; n = 10) and their daughters (Young cows, 1 to 4 year old; n = 10) during the month of June. A follicular wave was induced using estradiol-17β (5 mg; Catalog # E8875, Sigma Chemical Company, St. Louis, Missouri, USA) and progesterone (100 mg; Catalog # P0130, Sigma Chemical Company, St. Louis, Missouri, USA). Steroid hormones were dissolved in benzyl alcohol (0.4 mL; Catalog # B27354, BDH Inc., Toronto, Ontario, Canada), mixed with canola oil (2 mL; No Name®, Montreal, Quebec, Canada) and given intramuscularly. An intravaginal progesterone-releasing device (1.9 g progesterone; CIDR-B®, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) was inserted at the time of steroid treatment. Seven days after initial steroid treatment, a luteolytic dose of prostaglandin analogue (Cloprostenol 500 μg; Estrumate®, Schering Canada Inc., Pointe-Claire, Quebec, Canada) was given intramuscularly and the CIDR was withdrawn. A second treatment of estradiol-17β (1 mg) in canola oil was given intramuscularly 24 h after prostaglandin treatment to synchronize the preovulatory LH surge and ovulation, as previously described (Bo et al., 1994). All intramuscular treatments were given in semitendinosus muscle.

To record follicular development, transrectal ovarian ultrasonographic examinations were performed every 24 h by the same operator using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900, Instruments for Science and Medicine, Vancouver, British Columbia, Canada). Follicle diameters, relative location of follicles ≥4 mm in diameter and CL were recorded, as described previously (Pierson and Ginther, 1987; Baerwald et al., 2003a; Malhi et al., 2005). The

total numbers of 4 to 5 mm follicles in both ovaries were also counted to confirm a previous observation that old cows had fewer 4 to 5 mm follicles at wave emergence than young cows (Malhi et al., 2005). Blood samples were obtained every 12 h by jugular venipuncture in 10 mL heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey, USA) and centrifuged at 1500 X g for 15 minutes. The plasma was harvested and stored at -20°C until analysis.

4.2.2 Experiment 2: Ovarian Superstimulation

The experiment was conducted during the month of July with the same group of mother-daughter pairs used in Experiment 1; i.e., old cows (13-14 year old, n = 8) and young cows (1-4 year old, n = 7). Each cow completed one natural interovulatory interval between experiments 1 and 2. Transrectal ovarian ultrasonography was performed daily, as described in Experiment 1. Superstimulatory treatment was initiated at the time of naturally occurring ovulation (Day 0; expected time of wave emergence). Cows were given a total dose of 50 mg NIH-FSH-P1 units of porcine FSH/100 kg body weight divided bid for 4 days (i.e., 8 treatments of 6.25 mg NIH-FSH-P1 units of Folltropin®-V/100 kg body weight given in semitendinosus muscle at 12 h intervals; Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). The total FSH dose varied from 252 mg to 354 mg (311 ± 13 mg; mean ± SEM) in old cows; from 229 mg to 354 mg (301 ± 18 mg) in young cows, and was not different (P = 0.6) between age groups. All mother-daughter pairs were superstimulated with the same batch of Folltropin to eliminate possible variations in bioactivity and LH content.

Daily ultrasonography was performed from start of FSH treatment until 12 h after last FSH treatment to record the number of follicles in 4 to 5 mm, 6 to 8 mm and ≥9 mm

categories. Transvaginal ultrasound guided oocyte collection was performed 18 h after last FSH treatment for the purposes of a separate study. Blood samples were obtained every 12 h by jugular venipuncture. FSH treatments were given after obtaining blood samples. Plasma samples were processed as described for Experiment 1.

4.2.3 Hormone Radioimmunoassay

Plasma samples from mother-daughter pairs were analyzed in the same assay to distribute inter-assay variation equally between groups. FSH concentrations in all samples were measured using NIAMDD-anti-ovine FSH-1 primary antibody and expressed as USDA bovine FSH-I-l units (Evans et al., 1994; Honaramooz et al., 1998; Malhi et al., 2005). The range of the standard curve was 0.13 to 16 ng/mL. Using a sample volume of 200 μL, the assay has a minimum detection limit of 0.13 ng/mL (zero ligand versus 0.13 ng/mL, unpaired t-test, $P < 0.05$) (Chard, 1990). The intra- and inter-assay coefficients of variation were 6% and 10% for low reference samples (mean, 1.7 ng/mL) and 13% and 8% for high reference samples (mean, 3.8 ng/mL), respectively. Using a sample volume of 200 μL, the assay for circulating LH concentrations (Evans et al., 1994; Honaramooz et al., 1998; Malhi et al., 2005) had a minimum detection limit of 0.06 ng/mL with a standard curve ranging from 0.06 to 8 ng/mL. The intra- and inter-assay coefficients of variation for LH were 10% and 5% for low reference samples (mean, 0.4 ng/mL) and 5% and 4% for high reference samples (mean, 1.0 ng/mL), respectively.

Plasma progesterone concentrations were assayed in a solid-phase radioimmunoassay using a sample volume of 100 μL (Coat-A-Count®, Catalog number TKPG5, Diagnostics Products Corporation, Los Angeles, USA) with a minimum

detection limit of 0.1 ng/mL (Kastelic et al., 1999; Malhi et al., 2005). The intra-assay coefficients of variation for low, medium and high reference samples were 3%, 3%, and 4%, respectively. The inter-assay coefficients of variation were 7% for low reference samples (mean, 1.7ng/mL), 7% for medium reference samples (mean, 2.6 ng/mL) and 1% for high reference samples (mean, 11.7 ng/mL). For measurement of plasma estradiol concentration, a double-antibody radioimmunoassay kit was used with a plasma volume of 200 μL (Malhi et al., 2005) (Catalog number KE2D5; Diagnostics Products Corporation, Los Angeles, USA). This kit does not require estradiol extraction and the minimum detection limit of the assay was 1 pg/mL. The intra-assay coefficients of variation for low, medium and high reference samples were 8%, 9% and 6%, respectively. The inter-assay coefficients of variation were 5% for low reference samples (mean, 10.5 pg/mL), 6% for medium reference samples (mean, 16.3 pg/mL) and 10% for high reference samples (mean, 34.9 ng/mL).

4.2.4 Data Analysis

In Experiment 1, the dominant follicle of the induced wave was identified by retrospective analysis of ovarian follicular data. The dominant follicle was defined as the largest follicle of the wave and was first identified at 4 to 5 mm in diameter (Ginther et al., 1989a; Adams et al., 1994a; Jaiswal et al., 2004; Malhi et al., 2005). The day of follicular wave emergence was defined as the day when the dominant follicle was first detected at 4 to 5 mm in diameter (Ginther et al., 1989a; Adams et al., 1994a; Baerwald et al., 2003a; Jaiswal et al., 2004). Ovulation was detected by the disappearance of the dominant follicle that had been identified in a previous ovarian examination by transrectal ultrasonography (Baerwald et al., 2003a).

Serial data on circulating gonadotropins, steroid hormones, follicle numbers and diameter profiles of the dominant follicle were normally distributed, and were compared by analysis of variance for repeated measures (Proc Mixed; SAS Version 8.2 for MS Windows, SAS Institute Inc, Cary, North Carolina, USA), to determine the effects of age, day of treatment and interactions (Littell et al., 2000; Jaiswal et al., 2004). Data for plasma FSH concentration were centralized to peak concentrations and compared by analysis of variance for repeated measures to determine temporal relationships between the FSH surge and emergence of a new follicular wave. The numbers of 4 to 5 mm follicles were also centralized to wave emergence and compared by analysis of variance for repeated measures to assess the dynamics of follicle numbers during follicular wave emergence. Single-point, numerical data (e.g., Table 4.1) were compared between old and young cows by Student t-test and paired t-test. Values are expressed as mean ± SEM unless otherwise stated. Differences with P-values ≤ 0.05 were considered statistically significant; P-values between 0.05 and 0.10 were considered tendency towards a difference.

4.3 Results

4.3.1 Experiment 1: Ovarian Synchronization

4.3.1.1 Follicle wave development

The interval between initiation of steroid treatment and emergence of a new follicular wave was 4.3 ± 0.3 days and was not different (P = 0.8) between age groups (Table 4.1). As expected, peak numbers of 4 to 5 mm follicles in old and young cows were observed at the time of wave emergence (Fig. 4.2B and 4.2C). Old cows tended to have fewer 4 to 5 mm follicles at wave emergence than young cows (specific day comparison P = 0.07; Fig. 4.2C).

Table 4.1 Endocrine and follicular wave characteristics (mean ± SEM) of old cows (13 to 14 years old, n = 10) and their young daughters (1 to 4 years old, n = 9) during ovarian synchronization with estradiol and progesterone protocol (Experiment 1).

	Old Cows	Daughters	*P*-Value
Interval (days) between:			
Estradiol/progesterone treatment to FSH peak	3.6 ± 0.2	3.8 ± 0.3	0.5
Estradiol/progesterone treatment to wave emergence	4.2 ± 0.4	4.4 ± 0.3	0.8
Interval (h) between:			
Second estradiol treatment to LH surge	21.6 ± 1.6	14.0 ± 2.0	0.01
LH surge to ovulation	26.4 ± 1.6	34.0 ± 2.0	0.01
LH concentrations at the time of second estradiol treatment (ng/mL)	0.6 ± 0.1	0.8 ± 0.3	0.5

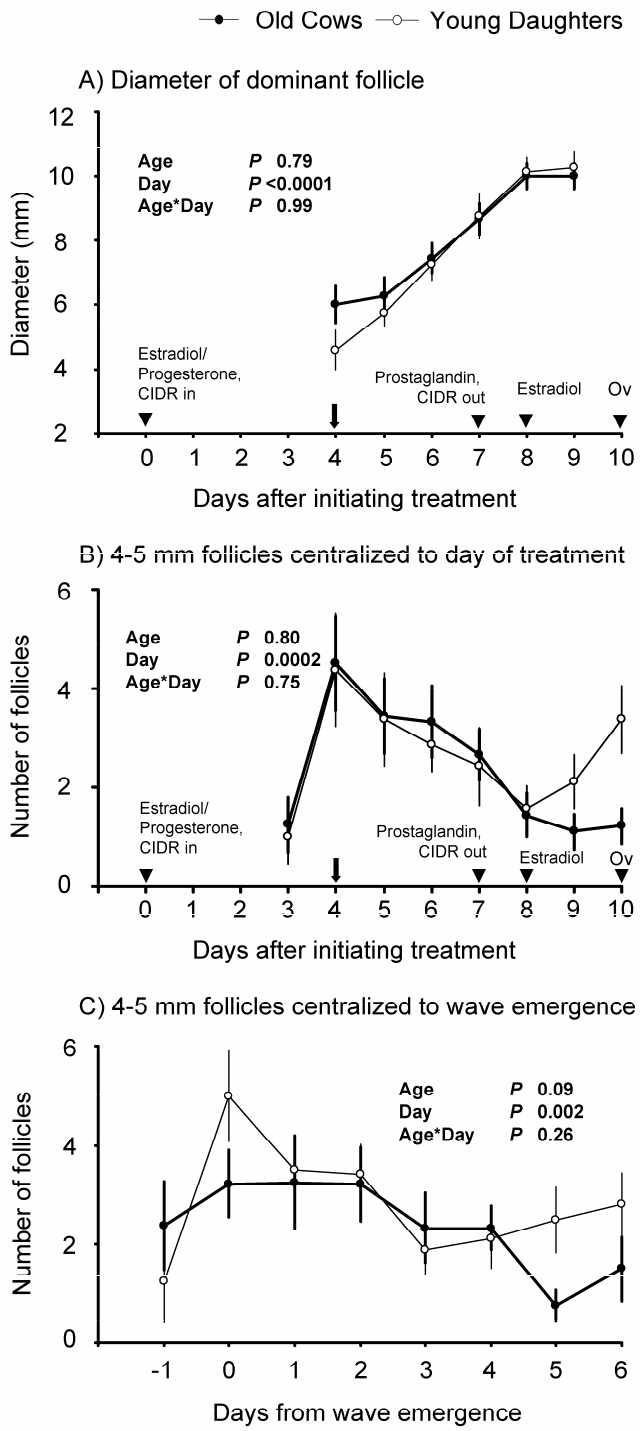

Figure 4.2 Diameter profiles of the dominant follicle relative to the day of treatment (A), and the number of 4 to 5 mm follicles (B, C) in old cows (n = 10) and their young daughters (n = 9) undergoing ovarian synchronization. An intravaginal progesterone-releasing device (CIDR) was maintained in place for 7 days. The emergence of a follicular wave is indicated by a black arrow, ovulation in all cows is indicated by Ov. Values are expressed as mean ± SEM.

In the follicular wave that emerged after steroid treatment, no differences were detected between old and young cows in the diameter profile of the dominant follicle (Fig. 4.2A). Ovulations were detected between 48 to 72 h after prostaglandin treatment in 19 of 20 cows (95%). One young cow did not respond to treatment and ovulated 192 h after prostaglandin treatment; hence, her data were not included in statistical analyses.

4.3.1.2 Gonadotropins

Greater mean circulating concentrations of FSH (P = <0.0001) were observed in old cows (0.7 ± 0.02 ng/mL) than young cows (0.5 ± 0.01 ng/mL; Fig. 4.3A). Steroid treatment on Day 0 resulted in suppression of FSH in both age groups (specific day comparison Pold = <0.0001 and Pdaughters <0.01; Fig. 4.3A). The interval between initial steroid treatment and the subsequent FSH peak was 3.7 ± 0.2 d and was not different between age groups (P = 0.5; Table 4.1). The FSH peak was also temporally associated with emergence of a new follicular wave (Fig. 4.3B). Plasma concentrations of LH did not differ between age groups during the treatment period (P = 0.8; Fig. 4.4A), or at the time of estradiol treatment on Day 8 (P = 0.5). The LH surge after estradiol treatment was delayed (P = 0.01) in old compared to young cows (Table 4.1), but no difference was detected in the interval from estradiol treatment to ovulation. There was no difference (P = 0.6) in the amplitude of the preovulatory LH surge in old cows (2.7 ± 0.8 ng/mL) versus young cows (2.1 ± 0.8 ng/mL).

4.3.1.3 Progesterone

Lower plasma progesterone concentrations were detected in old than young cows (P = 0.03; Fig. 4.4B). In both age groups, progesterone concentrations decreased to

<1 ng/mL within 24 h of luteolytic treatment. In the preovulatory period, there was no difference in plasma progesterone concentration between old and young cows.

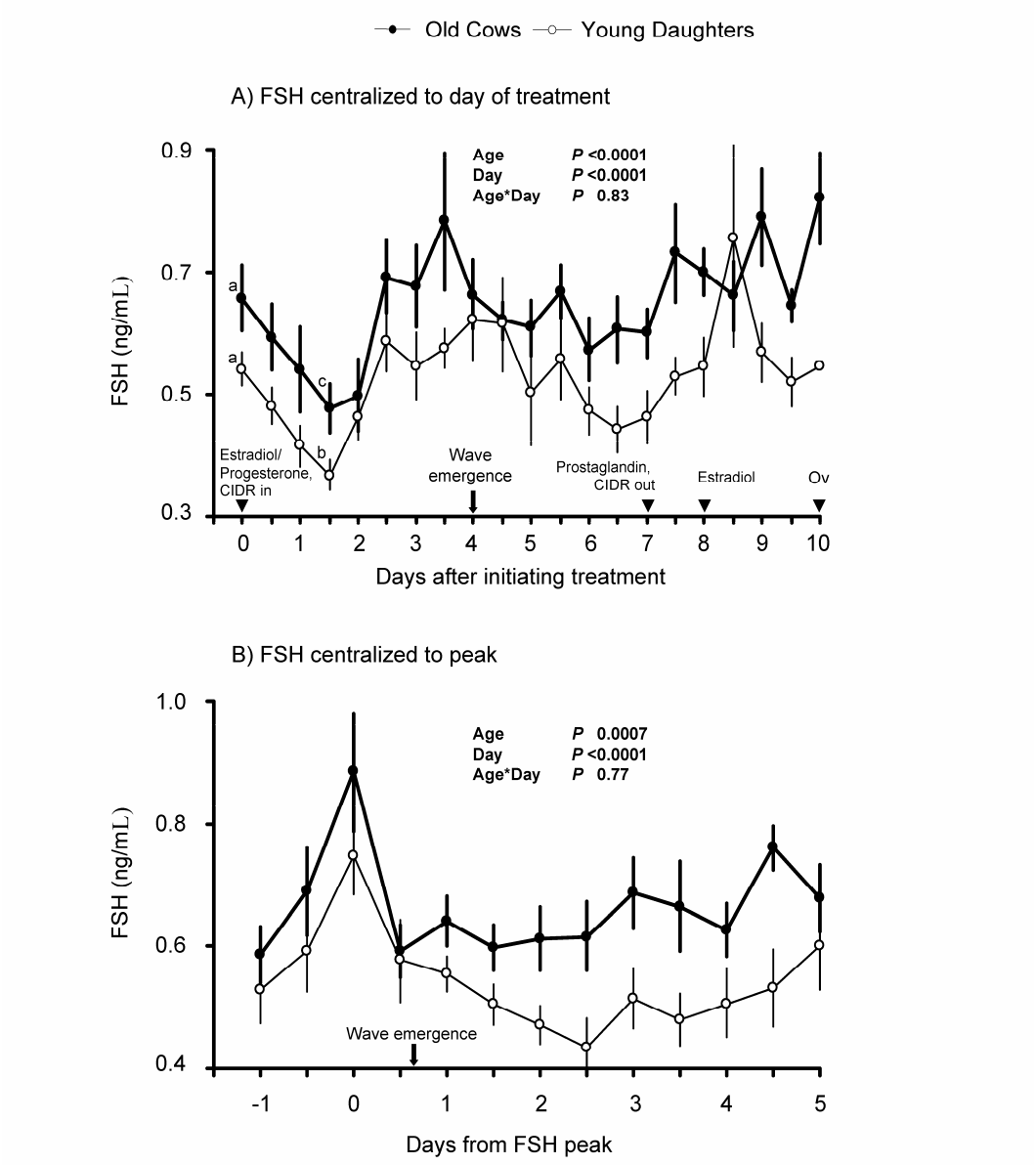

Figure 4.3 Plasma FSH concentrations (A, B; mean ± SEM) in old cows (n = 10) and their young daughters (n = 9) undergoing ovarian synchronization. An intravaginal progesterone-releasing device (CIDR) was maintained in place for 7 days. Ovulation in all cows is indicated by Ov. Values with no common superscripts are different ($P \leq 0.05$).

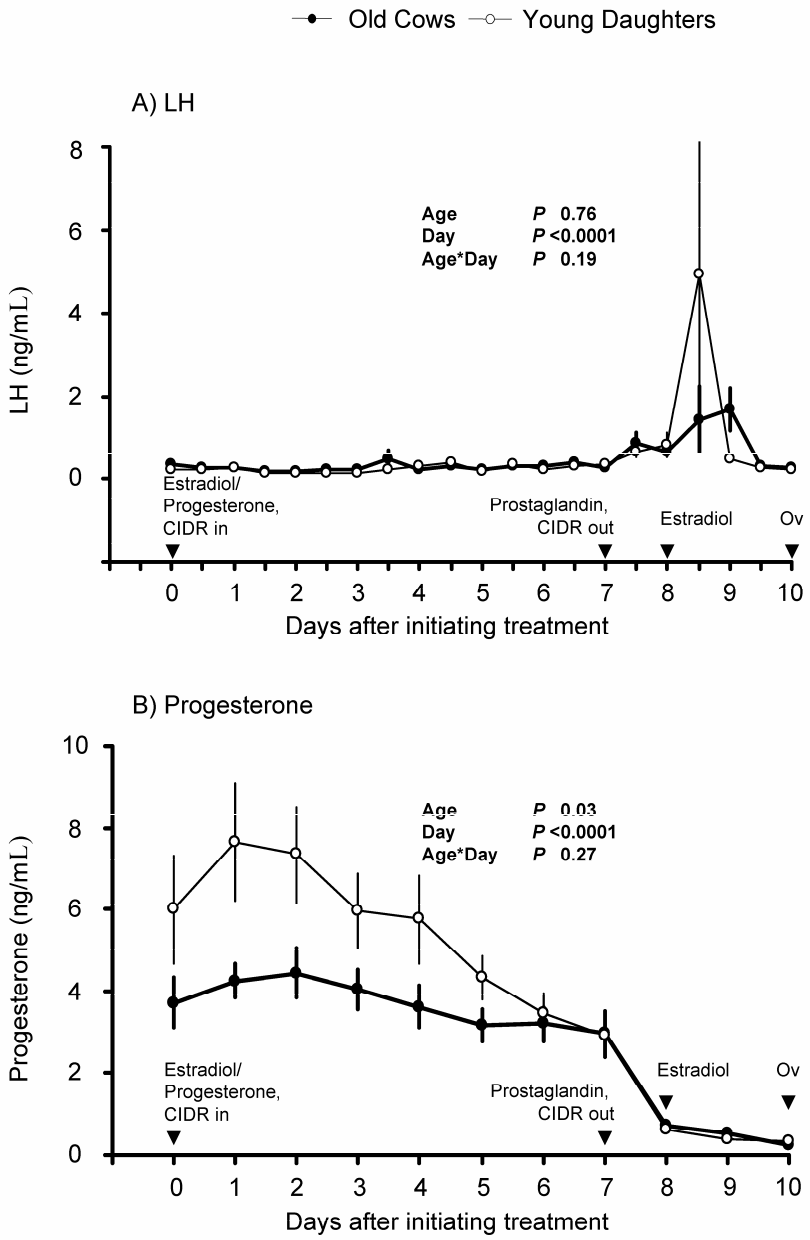

Figure 4.4 Plasma LH (A) and progesterone concentrations (B; mean ± SEM) in old cows (n = 10) and their young daughters (n = 9) undergoing ovarian synchronization. Data were analyzed relative to the day of treatment. An intravaginal progesterone-releasing device (CIDR) was maintained in place for 7 days. Ovulation is indicated by Ov.

4.3.2 Experiment 2: Ovarian Superstimulation

4.3.2.1 Follicle numbers

As expected, exogenous FSH treatments induced multiple follicle development. The number of 4 to 5 mm, 6 to 8 mm, ≥ 9 mm and total follicles (≥ 4 mm) did not differ between age groups (Fig. 4.5A-D), but a day effect ($P < 0.05$) on follicle numbers was observed in all categories. Old cows tended ($P = 0.10$) to have fewer (17.1 ± 3.3) large follicles (≥ 9 mm) 12 h after last FSH treatment than young cows (28.1 ± 5.5; specific day comparison $P = 0.09$; Fig. 4.5C). No ovulations were detected during the experimental period.

4.3.2.2 Gonadotropins

Age and day of treatment had no effect ($P = 0.2$) on plasma FSH concentrations (Fig. 4.6A) during the treatment period. Plasma LH concentrations during the treatment period did not differ between age groups (Fig. 4.6B). Relative to the first day of FSH treatment (day of ovulation, Day 0), a distinct LH surge was detected at 60.0 ± 0.0 h in old cows ($n = 5$) and at 56.0 ± 4.0 h in young cows ($n = 6$; $P = 0.4$). In the remaining three old cows and one young cow, no LH surge was detected. The amplitude of the LH surge did not differ between age groups (old cows, 3.0 ± 1.0 ng/mL versus young cows, 3.6 ± 0.9 ng/mL; $P = 0.7$).

4.3.2.3 Steroid hormones

The profile of circulating ($P = 0.1$; Fig. 4.7A) or peak ($P = 0.3$; Table 4.2) estradiol concentrations did not differ between age groups. The peak in plasma estradiol concentration tended to occur later ($P = 0.1$) in old cows than young cows (Table 4.2).

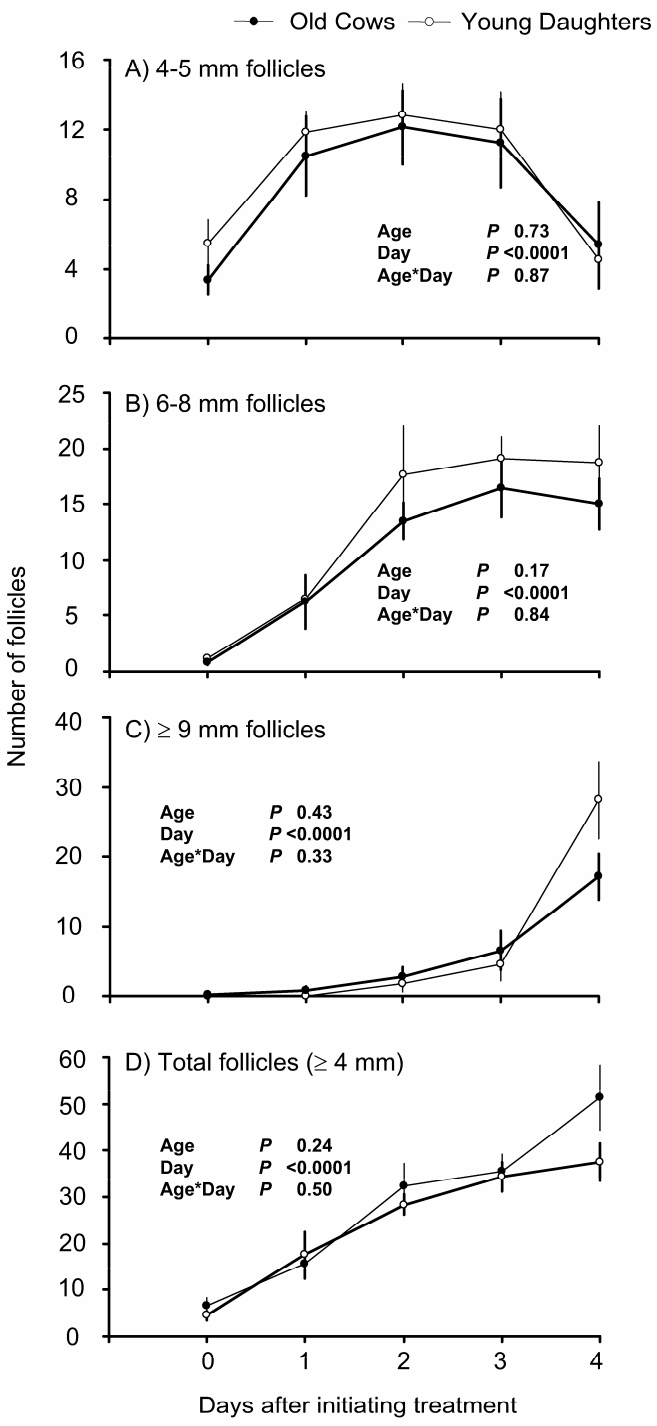

Figure 4.5 Number of follicles (mean ± SEM) in different diameter categories (A to D) in old cows (n = 8) and their young daughters (n = 7) undergoing ovarian superstimulatory treatment (FSH bid for 4 days).

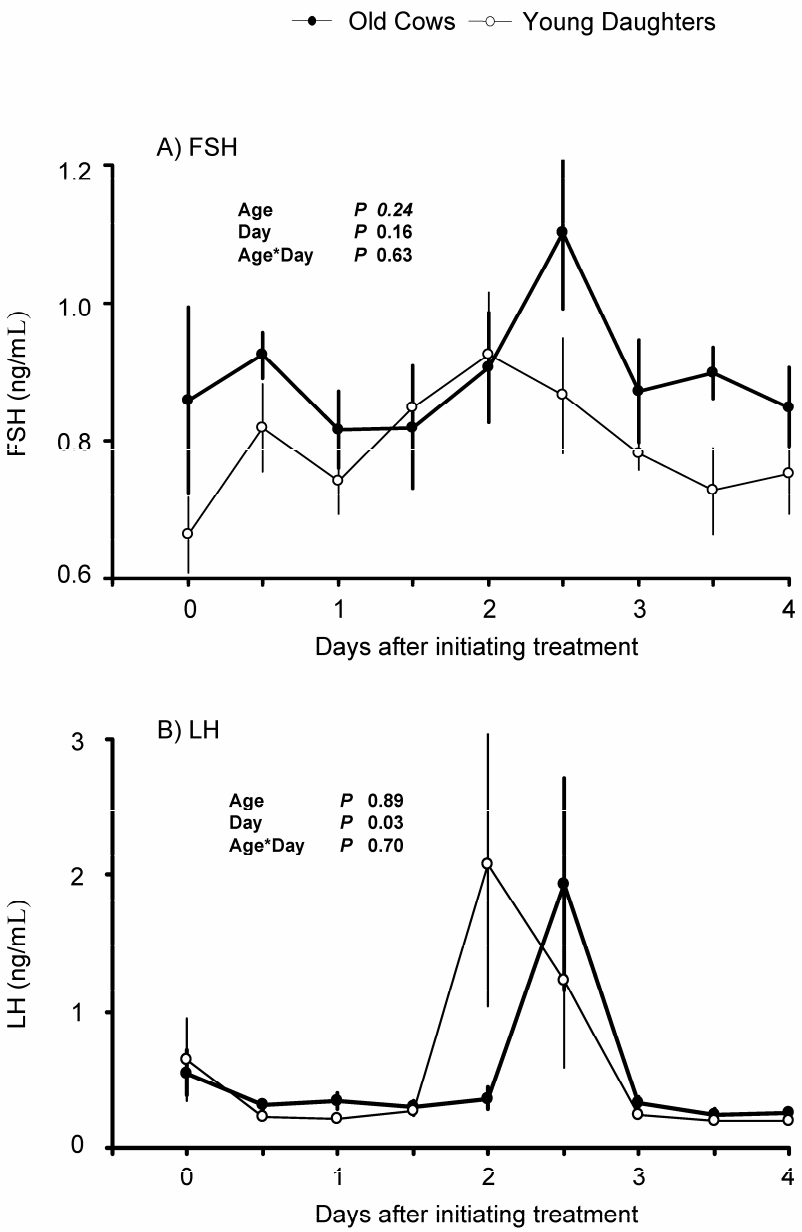

Figure 4.6 Plasma concentrations (mean ± SEM) of FSH (A) and LH (B) in old cows (n = 8) and their young daughters (n = 7) undergoing ovarian superstimulatory treatment (FSH bid for 4 days).

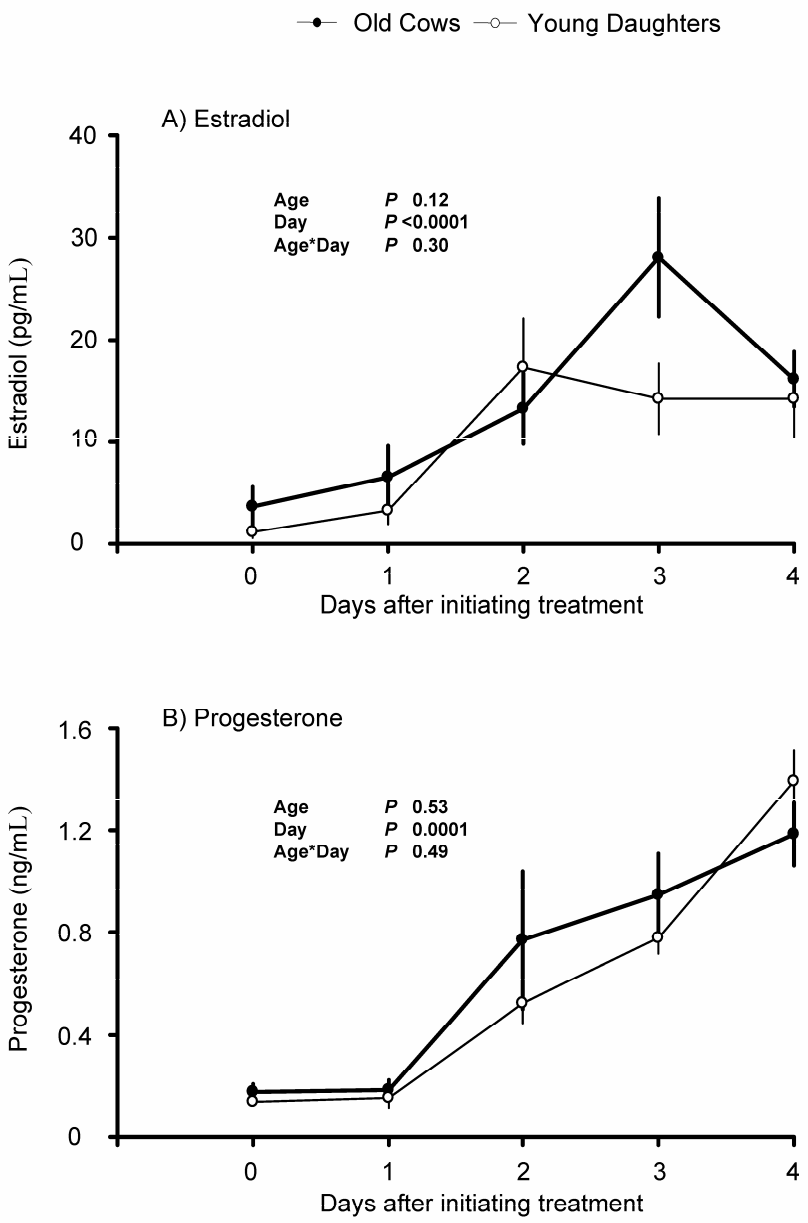

Figure 4.7 Plasma concentrations (mean ± SEM) of estradiol (A) and progesterone (B) in old cows (n = 8) and their young daughters (n = 7) undergoing ovarian superstimulatory treatment (FSH bid for 4 days).

Table 4.2 Endocrine characteristics (mean ± SEM) of old cows (13 to 14 year old, n = 8) and their young daughters (1 to 4 year old, n = 7) during ovarian superstimulation with exogenous porcine FSH given bid for 4 days (Experiment 2).

	Old Cows	Daughters	*P*-Value
Peak concentration[a]			
LH[b] (ng/mL)	3.0 ± 1.0	3.6 ± 0.9	0.7
Estradiol (pg/mL)	28.4 ± 5.4	21.2 ± 3.7	0.3
Interval from start of treatment to peak concentration (h)	60.0 ± 0.0	56.0 ± 4.0	0.4
LH[b]	75.0 ± 3.0	61.7 ± 7.1	0.1
Estradiol			

[a] Highest concentration detected after initiation of superstimulatory treatment
[b] Old cows, n = 5 and daughters, n = 6

As expected, low progesterone concentrations (< 1 ng/mL) were detected in both age groups to Day 3 of the treatment period (i.e., first 3 days after ovulation), followed by an increase (Day effect, P = 0.0001; Fig. 4.7B). Progesterone concentrations during experimental period did not differ between old and young cows (P = 0.5).

4.4 Discussion

Age-associated subfertility due to reduced developmental competence of oocytes is of increasing concern in veterinary and human medicine. A bovine model has been proposed for the study of ovarian aging in women (Malhi et al., 2005). Results from previous work in our laboratory have validated several important biological similarities between the two species in age-related ovarian and endocrine changes (Malhi et al., 2005). To further characterize this model, the present study was designed to examine the responsiveness of the aging hypothalamo-pituitary-gonadal axis to exogenous treatments for ovarian synchronization and superstimulation. Ovarian cycles are frequently manipulated in both cattle and women to obtain oocytes for the purposes of assisted reproductive technologies (Dew et al., 1998; Bo et al., 2002). We hypothesized that aging in cattle is associated with 1) decreased synchrony in FSH suppression after estradiol and progesterone treatment, with a subsequent decrease in synchrony of the FSH surge and follicular wave emergence, 2) delayed preovulatory LH surge and ovulation in response to exogenous estradiol treatment, and 3) a reduced superstimulatory response to exogenous FSH. The use of contemporaneous mother-daughter pairs allowed us to minimize the effects of genetic and environmental variations. The old cows used in this study represent the most fertile of the herd, as cows that fail to produce a calf are systematically culled.

Authors of previous studies (Bo et al., 1994; Bo et al., 2000) have demonstrated that treatment with estradiol and progesterone can effectively synchronize follicular wave emergence in cattle by suppressing circulating concentrations of gonadotropins. Treatment was followed by a rebound in circulating FSH and subsequent emergence of a new follicular wave 4 days later (Bo et al., 2002). In the present study, estradiol and progesterone treatment suppressed circulating FSH in both age groups for 36 h, and the intervals from treatment to the subsequent FSH peak (3.7 ± 0.2 d) and wave emergence (4.3 ± 0.3 d) were not different between old and young cows and were consistent with that of previous studies (Bo et al., 1994; Bo et al., 2002). Higher circulating concentrations of FSH were observed in old cows than young cows during ovarian synchronization in the present study; an observation that substantiates our previous findings in old cows (Malhi et al., 2005) and one that is consistent with the results of studies of reproductive aging in women (Klein et al., 1996b; Soules et al., 2001). Overall, results did not support the hypothesis that aging in this population of highly fertile cattle is associated with decreased hypothalamo-pituitary responsiveness to ovarian steroid treatment.

The preovulatory LH surge after estradiol treatment was delayed in old cows compared to young cows, although age groups did not differ in progesterone concentrations at the time of estradiol treatment. We did not detect a difference between age groups in the interval to ovulation which may be a reflection of insufficiently frequent ultrasound examinations (24 h intervals). The discrepancy between intervals to the LH surge, and to ovulation, warrants re-examination with more frequent ultrasonographic monitoring. Old cows had lower circulating progesterone during

ovarian synchronization than young cows which could be attributed to random stages of estrous cycles at the start of treatment.

A distinct LH surge, similar to a preovulatory surge, was detected 48 to 72 h after the start of superstimulatory treatment in five of eight old cows and six of seven young cows, and was attributed to increasing estrogen during a period of low circulating concentrations of progesterone (i.e., superstimulation immediately after natural ovulation when circulating progesterone was <1 ng/mL). Despite the LH surge, no ovulations were detected, likely because of the relatively small follicular diameters at 48 h after ovulation. No differences in peak estradiol concentrations between age groups were detected in this study, but peak concentrations tended to be delayed in old cows compared to young cows. Based on a 12 h sampling interval, the LH surge in three of six young cows was detected at 48 h after the start of treatment; the LH surge in all the old cows and remaining three young cows was detected 12 to 24 h later. Collectively, our results supported the hypothesis that aging in cattle is associated with a delayed preovulatory LH surge, but whether it is associated with delayed ovulation will require further study.

During ovarian synchronization, old cows tended to have fewer 4 to 5 mm follicles at induced wave emergence than young cows. This observation is consistent with previous findings that old cows had fewer 4 to 5 mm follicles at spontaneous follicular wave emergence than young cows (Malhi et al., 2005). Although the total number of follicles ≥4 mm did not differ between age groups during ovarian superstimulation, there were, on average, 11 fewer large follicles (≥9 mm) 12 h after the last FSH treatment in old cows than young cows. This observation is consistent with previous studies in which a reduced superovulatory response or reduced ova/embryo recovery was observed in aged

cattle (Lerner et al., 1986) and women (Kim, 1995; Dew et al., 1998). Studies in both women and cows document that the dose of gonadotropins influences the follicular response and the number of ova/embryos recovered (Lerner et al., 1986; Crosignani et al., 1989); however, old and young cows in the present study were given a consistent superstimulatory dose of FSH based on body weight, and had similar circulating concentrations of FSH, thus eliminating any possible dose effects. Study extending beyond the period of FSH treatment is required to determine if aging is associated with decreased follicular maturation and ovulation.

We have proposed a bovine model for study of ovarian function in women (Adams and Pierson, 1995). Validation for this model was provided by the discovery of follicular wave patterns and endocrine events in women (Baerwald et al., 2003a; b) similar to those observed in cattle (Ginther et al., 1989a; Adams, 1999) and horses (Ginther et al., 2004). More recently, we extended the bovine model for the study of reproductive aging in women (Malhi et al., 2005). Older cattle had higher circulating concentrations of FSH than their young daughters, analogous to women approaching the later stages of their reproductive years (Klein et al., 1996a; Malhi et al., 2005). Similarly circulating concentrations of FSH in present study were higher in old cows, and the pattern of FSH secretion in both age groups was similar before or after exogenous steroid treatment for ovarian synchronization. We observed that the response to ovarian synchronization treatment (synchrony of the FSH surge, follicular wave emergence, and or the diameter profile of the dominant follicle of the induced wave) was not affected in the cows in the 13- to 14-yr of age group. The pre-ovulatory LH surge was delayed in old cows, but the effect on interval to ovulation remains unclear. We speculate that fewer

follicles at wave emergence and fewer large follicles at the end of superstimulation in old cows compared to their young daughters may be attributed to 1) depletion of the ovarian follicular reserve, or 2) decreasing follicular sensitivity to FSH and LH. We propose that the bovine model may be used to develop quantitative and qualitative tests of the ovarian follicular reserve, and to develop strategies for reducing the effects of aging on ovarian superstimulation and fertility.

CHAPTER 5

SUPEROVULATION

Pritpal S. Malhi, Gregg P. Adams, Reuben J. Mapletoft and Jaswant Singh

5.1 Introduction

Development of ovarian follicular waves and methods to control wave emergence have been well characterized in cattle (Bo et al., 1995; Adams, 1999; Ginther et al., 2001; Mapletoft et al., 2002). A follicular wave has been defined as the synchronous growth of a group of 1-2 mm follicles that have been stimulated to grow by a surge of FSH (Adams et al., 1992b; Jaiswal et al., 2004). In the majority of bovine interovulatory intervals, either two or three waves of follicular development have been reported (Ginther et al., 1989a; Singh and Adams, 1998; Adams, 1999). Critically derived information on the effects of reproductive aging on follicular dynamics in cattle is sparse, but in a recent study (Malhi et al., 2005) of endocrine and follicular patterns in aged cows, circulating concentrations of FSH were increased and the number of small follicles recruited into each wave was decreased in old cows compared to their young daughters. However, the follicular wave pattern was maintained in 13-14-yr old cattle (Malhi et al., 2005).

The goal of ovarian superstimulation is to obtain multiple, developmentally competent oocytes for in vivo or in vitro embryo production. Exogenous FSH treatment for ovarian superstimulation initiated near the time of follicular wave emergence has been shown to prevent the selection of a single dominant follicle by preventing the regression

of subordinate follicles within the wave (Nasser et al., 1993; Adams et al., 1994b; Mapletoft et al., 2002). Using ultrasonography, the number of antral follicles ≥2 mm at the start of superstimulatory treatment was highly predictable of the superstimulatory response in cattle (Singh et al., 2004) and women (Ng et al., 2000).

The mechanisms of follicular wave emergence, selection of a dominant follicle, and ovulation were fundamentally similar in cattle and women (Adams and Pierson, 1995; Baerwald et al., 2003a). In recent studies in women, the majority of menstrual cycles were also composed of two or three follicular waves (Baerwald et al., 2003a; b). In all follicular waves of natural estrous cycles in cattle, and in major waves during the menstrual cycle in women, one follicle is selected to become dominant while the others (subordinates) regress (Adams et al., 1993; Ginther et al., 2001; Baerwald et al., 2003a). Furthermore, the earliest sign of reproductive aging in cattle (Malhi et al., 2005), and women (Klein et al., 1996a) was a rise in circulating concentrations of FSH. A decline in the antral follicle count (Scheffer et al., 1999), a reduced ovarian superstimulatory response, and lower subsequent ova/embryo recovery has been observed in older women (Kim, 1995; Dew et al., 1998). Similarly, old cows tended to have fewer ≥9 mm follicles after superstimulation (Malhi et al., 2006). Based on these similarities, a bovine model was proposed to study ovarian function and age-associated subfertility in women (Adams and Pierson, 1995; Malhi et al., 2005).

The present study was designed to test the hypotheses that aging in cattle is associated with 1) a reduction in the number of small antral follicle (2-5 mm) recruited into a wave, and 2) a reduced follicular and ovulatory response following gonadotropin treatment. In an early study, approximately 55% of the cows were classified as infertile

by 13 year of age (Erickson et al., 1976). However, old cows used in the present study were selected for fertility; i.e., other cows in the herd that had failed to produce a calf annually were culled. Thus, our group of 13-16 year old cows represents the top half of the herd in terms of fertility. To minimize the effect of inherent fertility in this group of cows, comparisons were made using their younger daughters.

5.2 Materials and Methods

The experiments were conducted in two consecutive years at the Goodale Research Farm, University of Saskatchewan, Saskatoon, SK, Canada (52° North and 106° West) during the months of May to July. The experiment was conducted in three to four replicates in both years, and mother-daughter pairs were superstimulated in the same replicate to distribute inter-replicate variation equally between age groups. Animals were at least 45 days post-partum, non-lactating, non-pregnant, and each had a corpus luteum at the beginning of the experimental period. All animals were maintained in single outdoor corral. The experimental protocol was approved by the University Committee on Animal Care and Supply under guidelines of the Canadian Council on Animal Care.

5.2.1 Experiment 1

The experiment was conducted on crossbred Hereford cows (13-16 year old, n = 6 in Year 1 and n = 9 in Year 2) and their young daughters (3-6 year old, n = 8 in Year 1 and n = 9 in Year 2). Mean (± SEM) body weights in old cows and their daughters were 737 ± 16 kg and 775 ± 29 kg, respectively. Estradiol-17β and progesterone treatment was used to induce the emergence of a new follicular wave (Bo et al., 1994; Bo et al., 1995; Malhi et al., 2006). Briefly, estradiol-17β (5 mg; Catalog # E8875, Sigma Chemical Company, St. Louis, Missouri, USA) and progesterone (100 mg; Catalog # P0130, Sigma

Chemical Company, St. Louis, Missouri, USA) were dissolved in benzyl alcohol (0.4 mL; Catalog # B27354, BDH Inc., Toronto, Ontario, Canada), mixed with canola oil (2 mL; No name®, Sunfresh Limited, Montreal, Quebec, Canada) and given intramuscularly. An intra-vaginal progesterone-releasing device containing 1.9 g progesterone (CIDR-B®, Pfizer Animal Health, Montreal, PQ, Canada) was inserted at the time of steroid treatment.

Ovarian superstimulation was induced using a standard 4-day FSH treatment protocol (Mapletoft et al., 2002) beginning at the expected time of follicle wave emergence; i.e., 4 days after estradiol 17-β and progesterone treatment (Bo et al., 1994; Mapletoft et al., 2002; Malhi et al., 2006). Cows were given a total dose of 50 mg NIH-FSH-P1 FSH/100 kg body weight im divided bid over 4 days (i.e., 8 treatments of 6.25 mg Folltropin-V®/100 kg body weight; Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). The total FSH dose (mean ± SEM) was 368 ± 8 mg in old cows, and 387 ± 14 mg in their daughters. On the last day of FSH treatment, 500 μg cloprostenol (Estrumate®, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) were given intramuscularly in the morning and evening, and CIDR were removed at the time of the second cloprostenol treatment. All cows were given 25 mg Armour standard LH (Lutropin-V®, Bioniche Animal Health Canada Inc.) 24 h after the second cloprostenol treatment to induce ovulation. All animals were artificially inseminated and embryos were collected for the purposes of a separate study.

To record follicular development, transrectal ovarian ultrasound examinations were performed every 24 h by the same operator using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900, Tokyo, Japan). Follicle numbers in

the 2-5 mm, 6-8 mm, 9-11 mm, and ≥12 mm categories were counted during each examination. The number of ovulations was estimated by the disappearance of follicles ≥6 mm recorded during the previous ultrasound examination. The cut-off of ≥6 mm diameter was chosen to minimize the possibility of missing any ovulations, and in a previous study, the diameter of the dominant follicles in superstimulated animals was smaller than single dominant follicle (Adams et al., 1993).

5.2.2 Experiment 2

The experiment was conducted after completion of Experiment 1 using the same group of old cows (13-16 year old, n = 7 in Year 1 and n = 9 in Year 2) and their young daughters (3-6 year old, n = 7 in Year 1 and n = 9 in Year 2). Mean (± SEM) body weights in old cows and their daughters were 738 ± 15 kg and 769 ± 30 kg, respectively. Sufficient time was allowed between experiments for each cow to complete one natural interovulatory interval. To induce ovulation thereafter, all cows in the luteal phase were given a single 500 μg dose of cloprostenol. The emergence of a new follicular wave was induced five to seven days after ovulation by transvaginal ultrasound-guided ablation of all follicles ≥5 mm (Bergfelt et al., 1997). Superstimulatory treatment with FSH was initiated at the expected time of wave emergence; i.e., 24 h after follicular ablation (Bergfelt et al., 1997; Mapletoft et al., 2002). Cows were given a total dose of 44 mg NIH-FSH-P1 FSH/100 kg body weight im divided bid for 3.5 days (i.e. 7 treatments of 6.25 mg Folltropin-V®/100 kg body weight). The total FSH dose (mean ± SEM) was 323 ± 7 mg in old cows, and 337 ± 13 mg in young daughters. Follicular development was recorded by transrectal ovarian ultrasound examinations as described in Experiment 1. For the purposes of a separate study, cumulus-oocyte complexes were aspirated from

follicles ≥5mm by transvaginal ultrasound-guided needle puncture 48 to 72 h after the last FSH treatment. Thus, superovulation data were not available from Experiment 2.

5.2.3 Data Analysis

Superstimulatory results (follicle data) from Experiments 1 and 2 were combined to determine the effects of age, year, and day of treatment. Serial data on follicle numbers in different diameter categories were analyzed by analysis of variance for repeated measures (Littell et al., 2000; Malhi et al., 2006) using the mixed procedure of the Statistical Analysis System (SAS version 8.2 for MS Windows, SAS Institute Inc, Cary, North Carolina, USA). Single point numerical data (e.g., follicle numbers, ovulations) were compared between old and young cows by paired t-tests, Student's t-tests, and the general linear model of SAS. Proportional data on ovulations were compared by chi-square analysis. Pearson's correlation for the number of follicles ≥6 mm (observed 5 days after the start of FSH treatment) between Experiments 1 and 2 and between Years 1 and 2 was performed using the correlation procedure in SAS. Similarly, the correlation between the numbers of ovulations in two years of Experiment 1 was calculated. Values are expressed as the mean ± SEM unless otherwise specified.

5.3 Results

The mean body weight and the total FSH dose given to old cows and to their young daughters in Experiments 1 and 2 were not different ($P > 0.3$). Fewer 2-5 mm follicles were detected at the time of initiation of FSH treatment (Day 0) in old cows than in their young daughters (23 ± 2 versus 28 ± 2, n = 29 mother-daughter pairs; $P = 0.006$). Fewer follicles were observed in old cows than in their daughters in the 6-8 mm, 9-11 mm and ≥12 mm categories ($P < 0.05$; Fig. 5.1B-D).

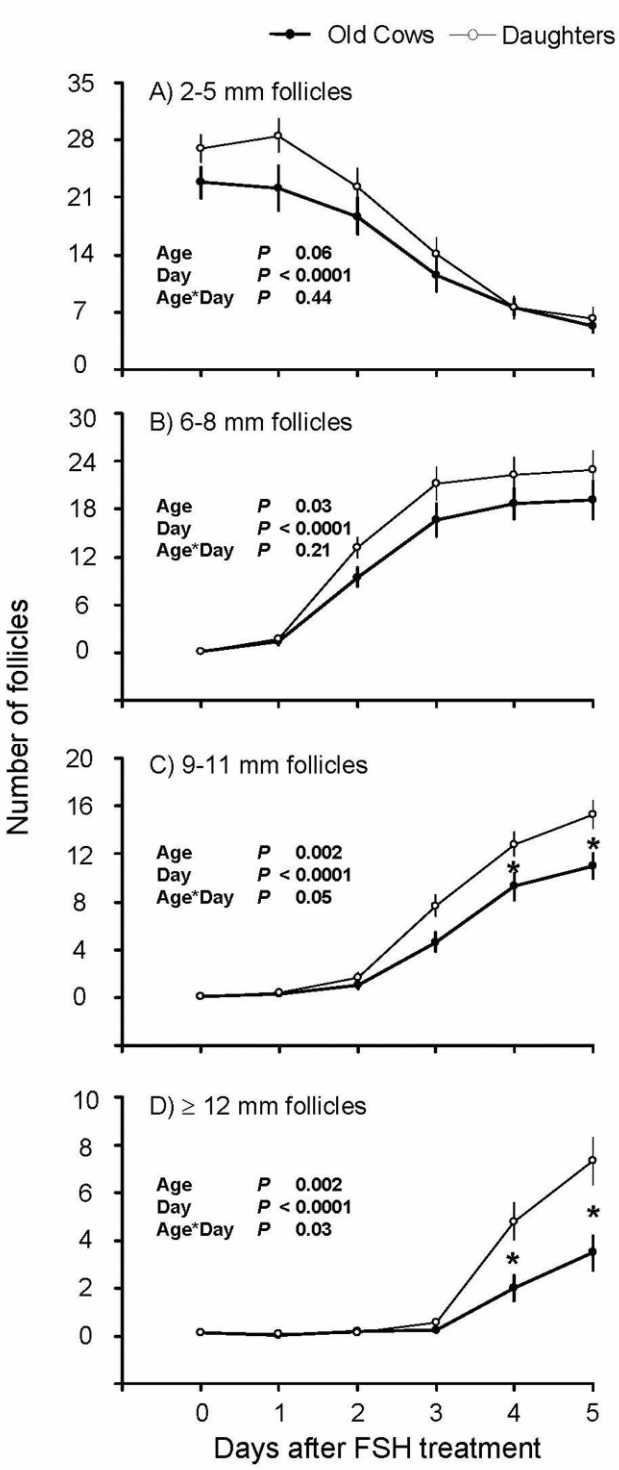

Figure 5.1 Number (mean ± SEM) of 2-5 mm follicles (A), 6-8 mm follicles (B), 9-11 mm follicles (C), and ≥12 mm follicles (D) in old cows (n = 31) and their young daughters (n = 33) undergoing ovarian superstimulation. Data were analyzed relative to the start of FSH treatment (Day 0). *Values between groups differ ($P \leq 0.05$).

The lower numbers of 9-11 mm and ≥12 mm follicles were attributed to differences on Days 4 and 5 (Age*Day interaction $P \leq 0.05$; Fig. 5.1C, D). Furthermore, fewer follicles ≥6 mm were observed on Day 5 in old cows than in their daughters (34 ± 3 versus 46 ± 3, n = 29 mother-daughter pairs; P = 0.0004).

When follicle number data from both experiments were compared between Years 1 and 2, more 2-5 mm follicles were detected in Year 2 than in Year 1 (P = 0.01; Fig. 5.2A). Similarly, more 6-8 mm follicles were observed on Days 4 and 5 of observational period in Year 2 than in Year 1 ($P < 0.05$, specific day comparison; Fig. 5.2B). Conversely, fewer 9-11 mm and ≥12 mm follicles were observed in Year 2 than in Year 1 ($P < 0.05$, specific day comparison; Fig. 5.2C, D). When numbers of 6-8 mm, 9-11 mm and ≥12 mm follicles on Day 5 were analyzed for age and year effect, there was no differential effect of year on follicle numbers in old cows and their daughters (age*year interaction, $P > 0.5$).

Data from Experiment 1 were also analyzed separately to study follicle numbers in relation to ovulations (Table 5.1). Most of the ovulations (84-94%) in old and young cows were detected between 24-48 h after LH treatment. The difference in the proportion of ovulations detected at different intervals (Table 5.1) was attributed primarily to one old cow in which most of the ovulations (Year 1 = 83%, Year 2 = 91%) were detected between 48-72 h after LH treatment. When data from this cow were excluded, the percentage of ovulations between 48-72 h after LH treatment did not differ between age groups (4% versus 4%). Irrespective of age, responses of individual cows (measured by number of ≥6 mm follicles detected on Day 5) after successive superstimulations were positively correlated between Years 1 and 2 ($r = 0.8$; $P < 0.0001$), and between

Experiments 1 and 2 (r = 0.9; P < 0.0001). Similarly, the number of ovulations within animal in Years 1 and 2 of Experiment 1 were also correlated (r = 0.6; P = 0.04).

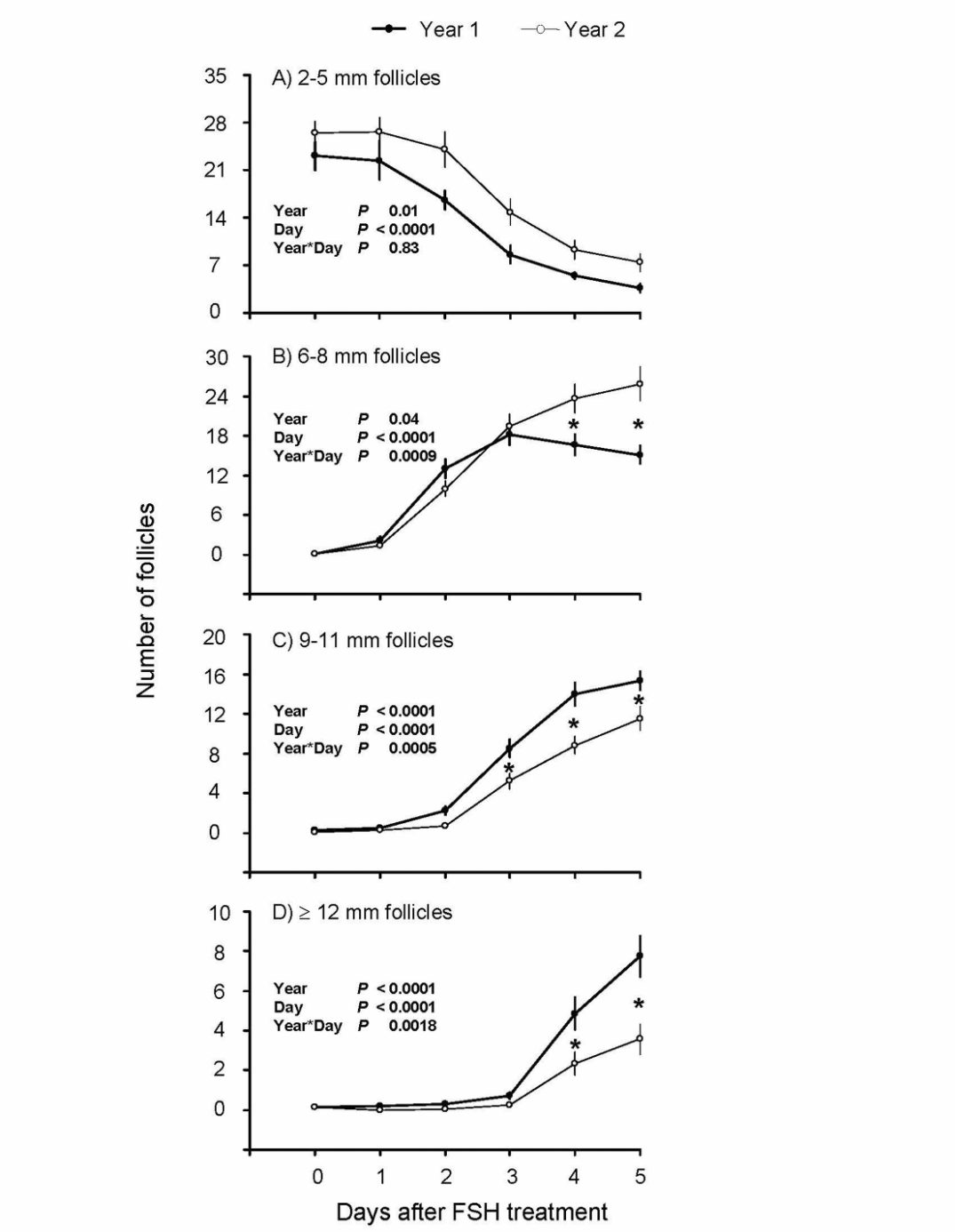

Figure 5.2 Number (mean ± SEM) of 2-5 mm follicles (A), 6-8 mm follicles (B), 9-11 mm follicles (C), and ≥ 12 mm follicles (D) in Year 1 (n = 28) and Year 2 (n = 36) undergoing ovarian superstimulation. Data were analyzed relative to the start of FSH treatment (Day 0). *Values between groups differ (P ≤ 0.05).

Table 5.1 Superovulatory data (mean ± SEM) from old cows and their young daughters (n = 14 mother-daughter pairs; Experiment 1).

	Old Cows	Daughters	*P*-Value
Number of :			
Follicles ≥ 6 mm 5 days after start of FSH treatment	39 ± 4	54 ± 4	<0.01
Ovulations	32 ± 4	40 ± 3	0.11
Proportion of:			
Ovulations from follicles ≥ 6 mm	0.81	0.74	0.24
Ovulations detected within 24 h of pLH treatment	0.03	0.02	0.10
Ovulations detected between 24 to 48 h after pLH	0.84	0.94	0.04
Ovulations detected between 48 to 72 h after pLH	0.13	0.04	0.04

5.4 Discussion

Over a decade ago, the bovine model was proposed for the study of ovarian function in women (Adams and Pierson, 1995), and the model formed the basis of recent discoveries of follicular wave dynamics in women (Baerwald et al., 2003a; b). Follicular and endocrine events of the normal reproductive cycle (i.e., follicular wave emergence, selection and ovulation) were fundamentally similar between cattle and women (Adams et al., 1992b; Adams and Pierson, 1995; Baerwald et al., 2003a; b). The first sign of reproductive aging in women was a rise in circulating concentrations of FSH (Klein et al., 1996a). Likewise, a rise in circulating FSH was detected in old cows (13-14 year) with no visible signs of infertility (Malhi et al., 2005). A reduction in the number of 4-5 mm follicles at the time of follicle wave emergence was also observed in the same group of aged cattle.

The present experiments were designed to study the effect of reproductive aging on recruitment of small (2-5 mm) antral follicles into follicular waves, and to compare follicular and ovulatory response in old cows and their young daughters. The mother-daughter pairs were used to reduce the effects of genetic variation. In support of the stated hypothesis, fewer 2-5 mm follicles were detected in old cows than in their young daughters at the expected time of follicular wave emergence. Consistent with a lower count of 2-5 mm follicles at wave emergence in the present study, significantly fewer 6-8 mm, 9-11 mm and ≥12 mm follicles were observed during the treatment period in old cows than in their daughters. Results were also consistent with other studies in which the antral follicle count (all follicles ≥2mm diameter) was highly predictive of the ovarian superstimulatory response in cattle (Singh et al., 2004) and in women (Ng et al., 2000).

Women with a low follicular response to ovarian superstimulation have been reported to show more signs of reproductive aging (Beckers et al., 2002), and enter into menopause earlier than those with a high follicular response within the same age groups (de Boer et al., 2002).

When combined over both experiments, fewer ≥6 mm follicles were detected at the end of superstimulatory treatment in old cows than in their young daughters. A similar effect was observed in a separate analysis of data from Experiment 1. The fewer number of follicles in old versus young cows was consistent with the tendency for a lower ovulation rate in older cows; on average, young cows had 8 more ovulations per cow than old cows. Thus, the hypothesis that reproductive aging is associated with a reduced follicular and ovulatory response after gonadotropin treatment was supported.

The proportion of ≥6 mm follicles that ovulated was not different when old cows and their daughters were compared, suggesting equal responsiveness to LH between age groups. There was a highly positive correlation in the response of individual cows to successive superstimulatory treatments, and the number of detected ovulations over years. Results are consistent with previous studies in cattle where the number of 2-6 mm follicles at follicular wave emergence was significantly correlated with their number in successive waves (Singh et al., 2004), and the repeatability index for the number of CL counted and the number of viable embryos recovered after ovarian superstimulation was 0.51 and 0.47, respectively (Peixoto et al., 2004). The repeatability for superovulatory response was also high in sheep (Bari et al., 2001), but is in contrast to a study in Holstein cattle (Tonhati et al., 1999) in which the repeatability of the superovulatory response, based on numbers of transferable embryos, was low (0.13).

In summary, reproductive aging in cattle was associated with a reduced number of small antral follicles at the time of follicular wave emergence, and a reduced superstimulatory and superovulatory response. The proportion of large follicles that ovulated was identical between age groups suggesting no age-related deterioration in the ovulatory mechanism. The previous superstimulatory and superovulatory response of individual cows was predictive of subsequent responses. Based on consistent age-related effects on ovarian function, the bovine model may be as useful for the study of reproductive senescence in humans as it has been for elucidating follicle dynamics during the natural menstrual cycle. In particular, the bovine model will aid in future studies of oocyte-associated subfertility in aging women (Malhi et al., 2005).

CHAPTER 6

OOCYTE DEVELOPMENTAL COMPETENCE

Pritpal S. Malhi, Gregg P. Adams, Reuben J. Mapletoft and Jaswant Singh

6.1 Introduction

Demographic analyses of monogamous populations that did not practice contraception revealed that fertility in women decreased with age (Tietze, 1957; Menken et al., 1986). However, these findings were confounded by decreased sexual activity with age, reproductive pathology associated with multiparity, and male infertility (Menken et al., 1986). These confounding variables were abrogated in a later retrospective study of 2193 nulliparous women whose husbands were sterile and who were artificially inseminated for 12 consecutive menstrual cycles using semen from fertile donors (Schwartz and Mayaux, 1982). Pregnancy rate in the women over 35 years of age was significantly lower (54%) than in the women below 31 years of age (74%). The results of more recent demographic studies (Chandra et al., 2005) and the national results of assisted reproductive technologies (Hull et al., 1996) substantiate the phenomenon of an age-related decline in female fertility.

Endocrine studies in older women demonstrated higher circulating concentrations of FSH compared to younger women (Klein et al., 1996a; Soules et al., 2001), attributed to reduced negative feedback as a result of lower circulating concentrations of inhibin B (Klein et al., 2004). Older women using assisted reproductive technologies to become pregnant also had lower ovarian follicular response to gonadotropin stimulation than

younger women, and embryos derived from oocytes of older women had lower implantation rates after transfer (ASRM, 2004). Studies involving oocyte donation from younger to older women are supportive of the notion that the age-related decline in fertility is due to reduced developmental competence of oocytes, and not differences in uterine receptivity in women of advanced age (Sauer, 1998). Oocyte chromosomal abnormalities, spindle defects and reduced mitochondrial function have been implicated in the age-related decline in fertility (Pellestor et al., 2003; Baird et al., 2005), but the mechanisms are not well understood. Research progress has been limited because it is difficult to obtain developmentally competent oocytes from women for hypothesis-based interventional studies, and most of the previous observations were made on oocytes that failed to develop into embryos after assisted reproductive cycles. Moreover, there is a lack of a well characterized animal model to study reproductive aging in women.

We proposed a bovine model to study ovarian function and the age-associated decline in fertility in women (Adams and Pierson, 1995; Malhi et al., 2005). As with women, the majority of interovulatory intervals in cattle are composed of either two or three waves of follicular development (Ginther et al., 1989a; Adams, 1999; Baerwald et al., 2003a; b). Furthermore, mechanisms of follicular wave emergence, selection of a dominant follicle, and ovulation were fundamentally similar (Adams and Pierson, 1995; Baerwald et al., 2003a). Age-related follicular and endocrine changes during a natural interovulatory interval have been documented in 13- to 14-yr-old cows, and were compared to their 1- to 4-yr-old daughters (Malhi et al., 2005). Old cows had elevated circulating concentrations of FSH (Malhi et al., 2005) similar to that of women (Klein et al., 1996a). The follicular wave pattern in older animals was similar to that of their young

daughters (Malhi et al., 2005). Old cows had fewer 4- to 5-mm follicles recruited into a follicular wave (Malhi et al., 2005), and had fewer large follicles after ovarian superstimulation (Malhi et al., 2006) when compared to their young daughters.

The present study was designed to test the hypothesis that aging in cattle is associated with reduced developmental competence of oocytes. We tested the hypothesis by comparing old cows and their young daughters in the 1) proportion of oocytes and embryos recovered after superovulation, artificial insemination and non-surgical uterine flushing, and 2) pregnancy rate and calves born after transfer of embryos into an unrelated group of young recipient cows.

6.2 Materials and Methods

The experiment was conducted at the Goodale Research Farm, University of Saskatchewan, Canada (52° North and 106° West) during the months of May and June in two consecutive years. Groups of crossbred Hereford cows (13-16 year old, n = 6; Year 1 and n = 9; Year 2) and their young daughters (3-6 year old, n = 8; Year 1 and n = 9; Year 2) were used as embryo donors. All cows were maintained together in an outdoor corral. Embryo donors were divided into three to four replicates in both years, but mother-daughter pairs were kept in the same replicate to minimize inter-replicate variation between age groups. A different group of young crossbred Hereford cows (2-5 year old, n = 32 in Year 1 and n = 67 in Year 2) were used as embryo recipients. The recipient cows were assigned to replicates based on age and body weight to minimize inter-replicate variability, and was maintained in outdoor corrals adjacent to the donors. All donor and recipient cows were at least 45 days post-partum, non-lactating, non-pregnant, and had a corpus luteum at the beginning of the study. The experimental protocol was

approved by the University Committee on Animal Care and Supply under guidelines of the Canadian Council on Animal Care.

6.2.1 Embryo Donors

The emergence of a new follicular wave was induced using a combined treatment of estradiol-17β (5 mg; Catalog # E8875, Sigma Chemical Company, St. Louis, Missouri, USA) and progesterone (100 mg; Catalog # P0130, Sigma Chemical Company, St. Louis, Missouri, USA) given intramuscularly at random stages of the estrous cycle (Malhi et al., 2005; Malhi et al., 2006). An intravaginal progesterone-releasing device (1.9 g progesterone; CIDR-B®, Bioniche Animal Health., Belleville, Ontario, Canada) was inserted at the time of steroid treatment. Ovarian superstimulatory treatment was initiated at the time of expected follicle wave emergence (i.e., four days after estradiol/progesterone treatment (Bo et al., 1994; Mapletoft et al., 2002)), using an intramuscular dose of 50 mg NIH-FSH-P1 porcine FSH/100 kg body weight (6.25 mg Folltropin-V®/100 kg body weight; Bioniche Animal Health, Belleville, Ontario, Canada) twice daily for four days. On the last day of FSH treatment, a luteolytic dose of cloprostenol (500 μg Estrumate®, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) was given intramuscularly in the morning and evening, and the CIDR-B was removed at the time of the second cloprostenol treatment. An ovulatory dose of porcine LH (25 mg Armour standard, Lutropin-V®, Bioniche Animal Health, Belleville, Ontario, Canada) was given intramuscularly 24 h after the second cloprostenol treatment. Cows were artificially inseminated 12 and 24 h after LH treatment by one inseminator using frozen semen of the same ejaculate of a single bull. Cows with unovulated follicles detected 12 h after the second insemination was inseminated again.

Daily transrectal ovarian ultrasound examinations were done by one operator using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900, Tokyo, Japan) to record follicular development and ovulations, beginning at the time of estradiol/progesterone treatment. Follicle numbers in the 2 to 5 mm and ≥6 mm diameter categories were counted during each examination. The number of ovulations was estimated by the disappearance of follicles ≥6 mm recorded during the previous ultrasound examination.

Oocytes and embryos were recovered seven days after the first insemination by non-surgical uterine flushing using Vigro® complete flushing medium (Bioniche Animal Health, Belleville, Ontario, Canada). Two experienced operators performed all embryo collections, but each mother-daughter pair was flushed by the same operator to minimize within-pair variation. The recovered embryos were graded by a single, experienced embryo transfer practitioner based on the morphological classification of International Embryo Transfer Society (IETS) (Stringfellow and Seidel, 1998). Unfertilized oocytes were not distinguished from uncleaved zygotes as sperm penetration could not be evaluated reliably seven days after insemination. Ovarian ultrasound examination of donor animals was also performed on the day of embryo collection to count the number of corpora lutea.

6.2.2 Embryo Recipients

Follicular wave emergence was synchronized using estradiol-17β, progesterone and CIDR-B treatment, as described for donor cows (Malhi et al., 2005; Malhi et al., 2006), except that the recipients were treated one day before the donors. A luteolytic dose of cloprostenol (500 μg Estrumate®, Schering-Plough Animal health, Pointe-Claire,

Quebec, Canada) was given intramuscularly at the time of CIDR removal, on the evening of the seventh day after estradiol/progesterone treatment. Recipients were given a second intramuscular dose of estradiol-17β (1 mg in canola oil) 24 h after prostaglandin treatment and CIDR-B removal to synchronize the preovulatory LH surge and ovulation (Malhi et al., 2006).

6.2.3 Embryo Transfers

Embryos were transferred transcervically into the uterine horn ipsilateral to the corpus luteum by one experienced operator. In year 1, most of the embryos were transferred as pairs; i.e., 56 embryos into 28 recipients. Four embryos were transferred as singletons. In year 2, 52 embryos were transferred as pairs into 26 recipients, and 41 embryos were transferred as singletons. Most of the embryos transferred were of IETS Grades 1 and 2, but three Grade 3 embryos were also transferred.

6.2.4 Embryo Survival/Loss

Embryo survival/loss in recipient animals was determined by transrectal ultrasonographic examination 28 to 30 days and again 45 to 48 days after donor insemination, and by the number of calves born.

6.2.5 Data Analysis

Ovulation, corpus luteum and ova/embryo recovery data were compared between old and young cows by Student's t-test. Pearson's correlation coefficient was determined between numbers of ovulations and corpora lutea using the correlation procedure of SAS (Statistical Analysis System for windows version 9.1.3, SAS Institute Inc, Cary, North Carolina, USA). The proportional data on ova/embryo recovery and embryo survival were compared between old and young cows by chi-square analysis or Fisher's Exact test

using the frequency procedure of SAS. A probability value of ≤ 0.05 was considered statistically significant.

6.3 Results

The superstimulatory dose of FSH, the number of ovulations and corpora lutea in both year 1 and 2 did not differ between old cows and their young daughters (Table 6.1). When data were combined between age groups and years, there was a significant correlation ($r = 0.85$, $P < 0.0001$) between the number of ovulations and corpora lutea detected by ultrasonography.

Inability to catheterize the cervix of one young daughter in year 2 precluded uterine flushing; hence, her data were not included in analysis of oocyte/embryo recovery rate. The total number of oocytes and embryos recovered did not differ between old cows and their daughters (Table 6.1). When combined over both years, there were significantly ($P = 0.04$) higher mean number of oocytes, and fewer mean number of embryos recovered from old cows than their daughters (Table 6.1). When oocyte and embryo data from all animals of each age group were combined, a higher proportion of oocytes and a lower proportion of embryos were recovered from old cows than their young daughters ($P < 0.0001$, Fig. 6.1A). Among the recovered embryos, the proportion of Grades 1 and 2 embryos did not differ ($P > 0.5$) between old cows and their young daughters (Fig. 6.1B).

The mean of proportion of oocytes collected per cow was higher ($P = 0.05$) in old cows (66 ± 9%, n = 15) than in their young daughters (41 ± 8%, n = 16). Two old cows in years 1 and 2, and two young cows in year 1 produced only oocytes, no embryos. When data from these cows were excluded from the analysis, the mean of proportion of oocytes tended ($P = 0.08$) to be higher in old cows (54 ± 10%, n = 11) than in their young daughters (33 ± 7%, n = 14).

Table 6.1 Ovarian superstimulatory dose of FSH, and the superovulatory response in old cows (13 to 16 years old) and their young daughters (3 to 6 years old). The values are expressed in mean ± SEM.

	Old Cows	Young Daughters	*P*-Value
Total FSH dose in mg NIH-FSH-P1 units			
Year 1	381 ± 15 (n = 6)	388 ± 16 (n = 8)	0.8
Year 2	360 ± 9 (n = 9)	387 ± 24 (n = 9)	0.3
Year 1 and 2 combined	368 ± 8 (n = 15)	387 ± 14(n = 17)	0.3
Numbers of ovulations detected by ultrasonography			
Year 1	30 ± 5 (n = 6)	33 ± 2 (n = 8)	0.5
Year 2	32 ± 6 (n = 9)	43 ± 5(n = 9)	0.1
Year 1 and 2 combined	31 ± 4 (n = 15)	38 ± 3(n = 17)	0.2
Numbers of corpora lutea on the day of embryo recovery			
Year 1	23 ± 3 (n = 6)	25 ± 1 (n = 8)	0.5
Year 2	26 ± 5 (n = 9)	32 ± 4(n = 9)	0.1
Year 1 and 2 combined	25 ± 3 (n = 15)	29 ± 2 (n = 17)	0.3
Oocyte and embryo recovery			
Year 1	22 ± 5 (n = 6)	17 ± 4 (n = 8)	0.5
Year 2	20 ± 5 (n = 9)	23 ± 3(n = 8)	0.7
Year 1 and 2 combined	21 ± 4 (n = 15)	20 ± 3 (n = 16)	0.8
Embryo recovery			
Year 1	4 ± 2 (n = 6)	9 ± 3 (n = 8)	0.2
Year 2	7 ± 3 (n = 9)	16 ± 4(n = 8)	0.08
Year 1 and 2 combined	6 ± 2 (n = 15)	12 ± 2 (n = 16)	0.04
Oocyte recovery*			
Year 1	18 ± 5 (n = 6)	8 ± 2 (n = 8)	0.07
Year 2	12 ± 4 (n = 9)	7 ± 2(n = 8)	0.2
Year 1 and 2 combined	15 ± 3 (n = 15)	7 ± 2 (n = 16)	0.04

*Includes unfertilized oocytes and zygotes that did not cleave

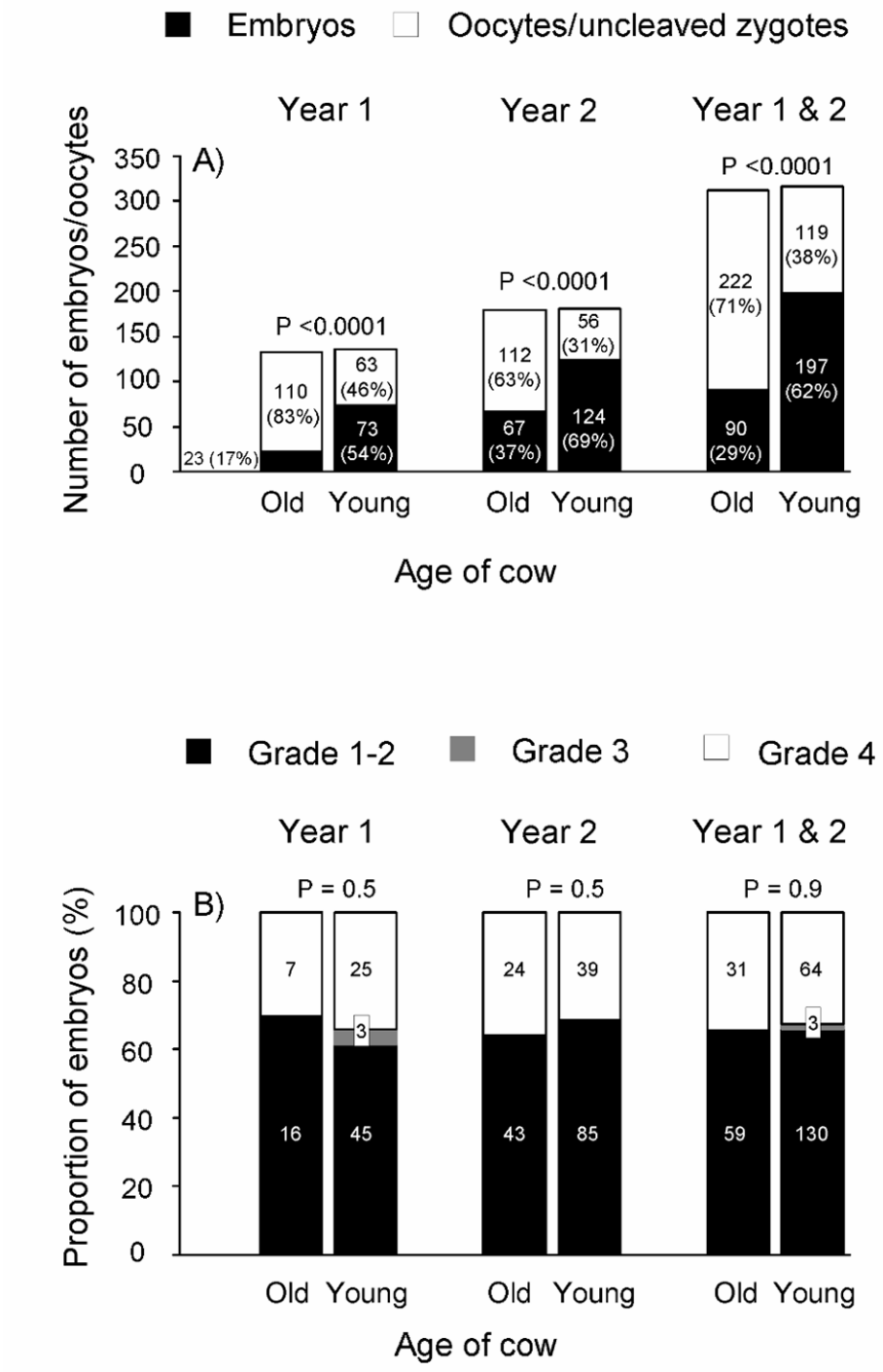

Figure 6.1 Numbers of oocytes and embryos collected (A) and the relative proportions of embryos by IETS grade (B) from old cows (n = 6 and 9 in Years 1 and 2, respectively) and their young daughters (n = 8 in each of Years 1 and 2).

A higher proportion (P = 0.03) of old cows (10/15; 67%) had > 50% oocytes collected, of the total oocytes/embryos recovered, than their young daughters (4/16; 25%).

The survival of embryos after transfer from young and old cows is summarized in Figure 6.2. The survival of embryos obtained from old cows versus their young daughters (Fig. 6.2) did not differ. The survival of embryos transferred into recipient cows as singletons versus pairs did not differ, when combined between age groups and years (Fig. 6.3). Pregnancy losses after 28 days post-insemination did not differ between old and young cows (7/24; 29% versus 10/49; 20%, respectively; Fig. 6.2), when combined for both years. However, fetal loss between 45 days post-insemination and calving tended to be higher (P = 0.08) in old cows than their young daughters (6/23; 26% versus, 4/43; 9%, respectively; Fig. 6.2).

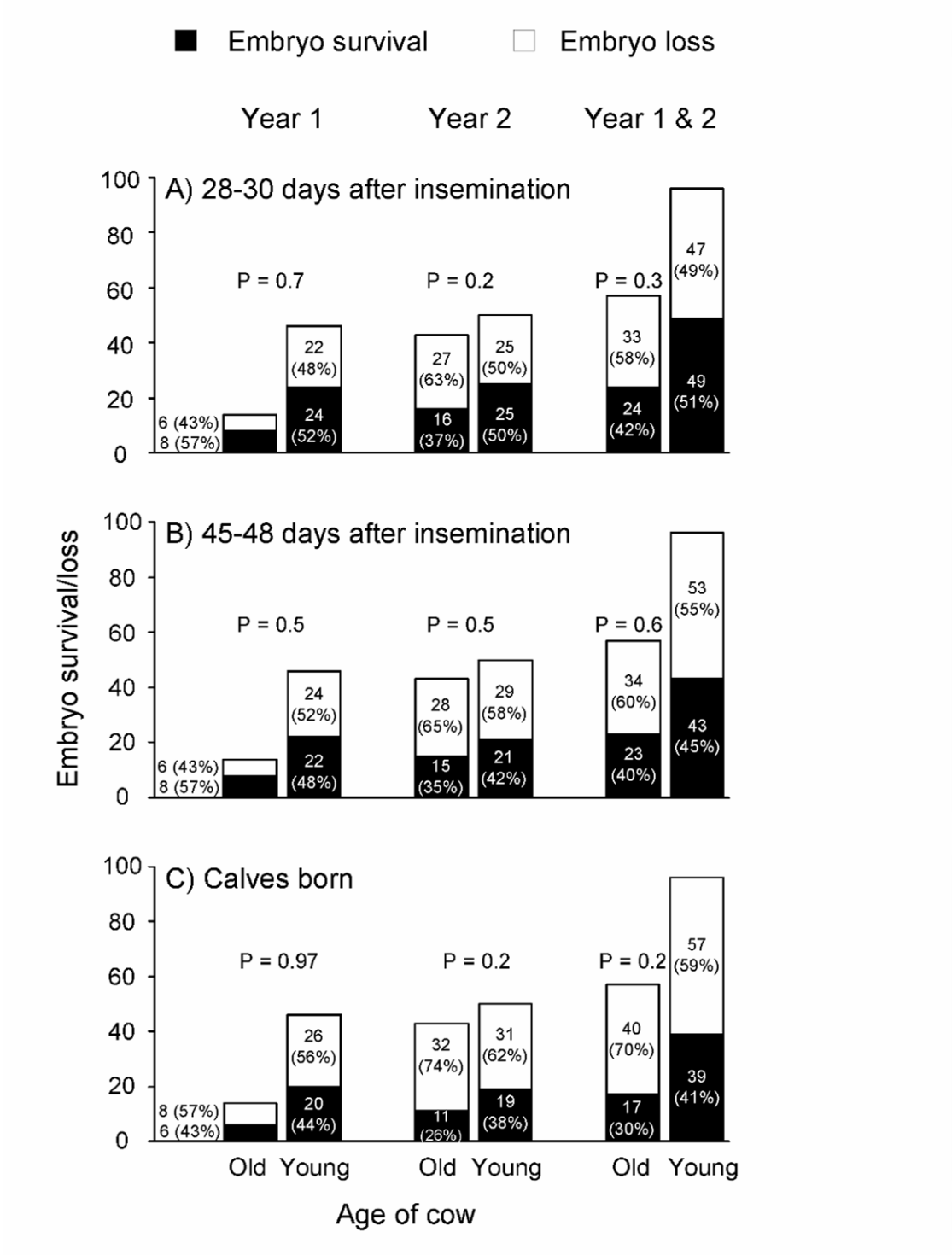

Figure 6.2 Survival of embryos recovered from old cows (n = 6 and 9 in Years 1 and 2, respectively) and their young daughters (n = 8 in each of Years 1 and 2) after transfer to recipient cows (n = 32 and 67 in Years 1 and 2, respectively), detected by ultrasonography at 28-30 days post-insemination (A), 45-48 days post-insemination (B), and by live-born calves (C).

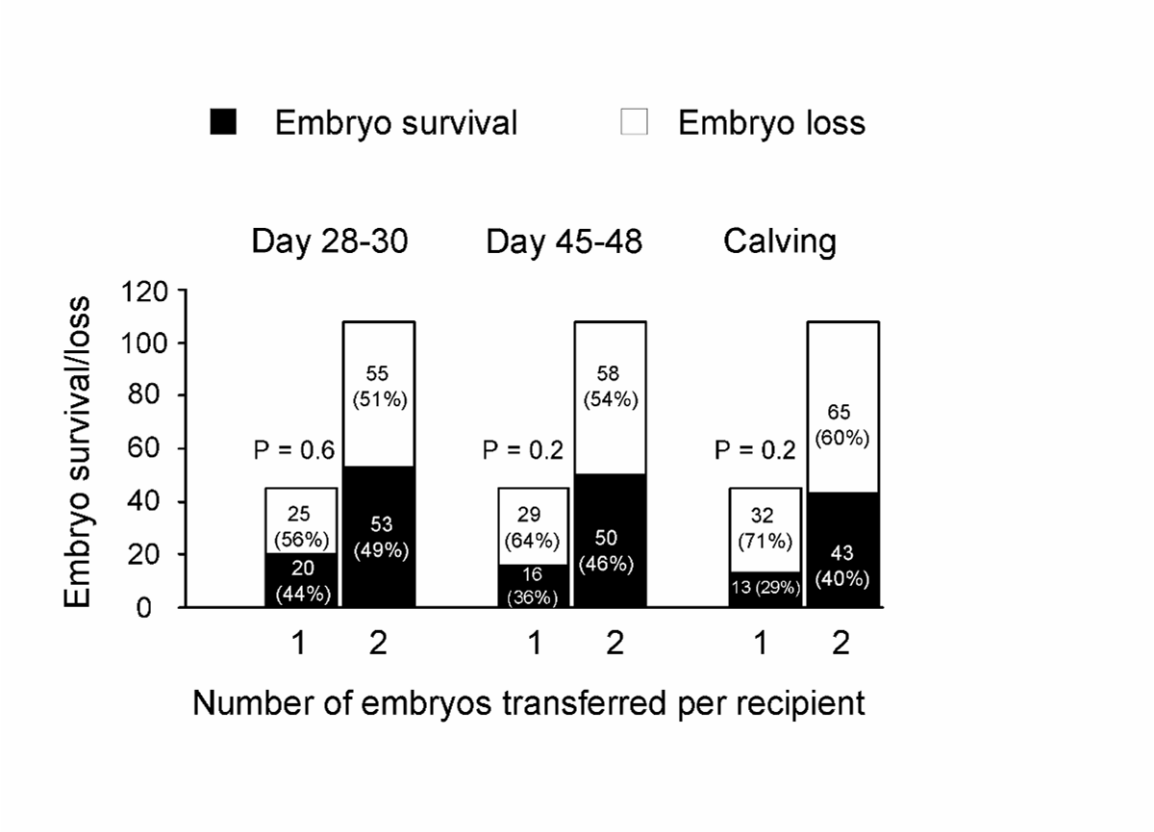

Figure 6.3 Embryo survival after transfer of either 1 or 2 embryos into each recipient cow (n = 32 and 67 in Years 1 and 2, respectively), detected by ultrasonography at 28-30 days post-insemination, 45-48 days post-insemination, and by live-born calves.

6.4 Discussion

Retrospective analyses of demographic records have documented a decline in fertility in women over 35 years of age (Tietze, 1957; Menken et al., 1986; Chandra et al., 2005). In addition, the success rates of donor insemination, in vitro fertilization and embryo transfer (IVF-ET) in women also decreased with age (Schwartz and Mayaux, 1982; Hull et al., 1996; Dew et al., 1998; Chandra et al., 2005). Following ovarian superstimulation for assisted reproduction, fewer oocytes were recovered, the embryo implantation rate after IVF-ET was lower, gestational attrition was higher, and the incidence of chromosomal abnormalities was greater in women over 35 years of age than in younger women (Hull et al., 1996; Spandorfer et al., 2004). Higher circulating concentrations of FSH have also been observed with advancing age in women (Klein et al., 1996a; Soules et al., 2001), but the mechanisms involved in the age-related decline in fertility are not well understood, and there is a lack of a well characterized animal model to study reproductive aging.

A bovine model to study ovarian function in women has been proposed (Adams and Pierson, 1995), and was the basis of the discovery of follicular waves in women (Baerwald et al., 2003a; b). Follicular and endocrine events of the normal reproductive cycle; i.e., follicular wave emergence, follicle selection, and ovulation, were fundamentally similar between cattle and women (Adams et al., 1992b; Adams and Pierson, 1995; Baerwald et al., 2003a; b). The first sign of reproductive aging in women was a rise in circulating concentrations of FSH (Klein et al., 1996a). A similar rise in circulating FSH was also detected in aging cows (13-14 year old) (Malhi et al., 2005). Fewer small ovarian follicles at the time of follicle wave emergence, and fewer large follicles after ovarian superstimulation were also observed in the same group of aged

cattle when compared to their young daughters (Malhi et al., 2006). Based on these findings, we proposed a bovine model for the study of oocyte-associated subfertility in women of advanced age (Malhi et al., 2005).

The present experiment was designed to test the hypothesis that aging in cattle is associated with reduced developmental competence of oocytes. In an early herd-based study, about half of the cows were infertile by 13 year of age (Erickson et al., 1976). Therefore, we chose 13- to 16-yr-old cows to test our hypothesis, while their 3- to 6-yr-old young daughters were used for comparisons. It is noteworthy that old cows in the present study were selected for fertility; i.e., other cows in the same herd that failed to produce a calf annually were culled. Age-related follicular and endocrine changes during a natural interovulatory interval were documented previously in the same groups of animals (Malhi et al., 2005). To test the stated hypothesis, data from old cows and their young daughters were analyzed by comparing 1) the actual number of recovered oocytes/embryos, 2) the proportion of total oocytes/embryos recovered in each age group, 3) the proportion of oocytes/embryos recovered from each individual, and 4) the pregnancy rate and number of calves born after transfer of embryos recovered from old and young cows into an unrelated group of young recipients. The recovery of fewer embryos and a higher proportion of oocytes collected from aged cattle, compared to their young daughters, suggest that fertilization or cleavage rates decline with age. This conclusion is supported by the observation that of the total oocytes/embryos recovered per cow, significantly more old cows (10/15, 67%) produced > 50% oocytes compared to their young daughters (4/16, 25%).

Although the experiment was not designed to directly test gamete transport among age groups, oviductal transport of oocytes/embryos was similar (P = 0.11) between age groups based on the number of oocytes/embryos recovered as a proportion of detected ovulations (66 ± 6% in old cows and 53 ± 6% in young daughters). Analysis of oocyte/embryo recovery by including data from only those animals that produced at least two or more embryos (i.e., confirming the presence of spermatozoa in the oviducts) revealed a higher (P = 0.05) proportion of oocytes and/or uncleaved zygotes in old cows (50 ± 10%, n = 10) than in their daughters (28 ± 5%, n = 13).

In a retrospective analysis of data from commercial embryo transfers in Holstein cows (Lerner et al., 1986), a significant decrease in fertilization rate was also observed with advancing age, consistent with the findings of the present study. In another study (Fujino et al., 1996), ovulated oocytes recovered from the oviducts of aged mice had a lower fertilization rate in vitro (determined by counting zygotes at the 2-cell stage) when compared to those from young mice. Contradictory results have been obtained from IVF studies in women; some reported reduced fertilization/cleavage rates in women >40 years of age (Ashkenazi et al., 1996; Dew et al., 1998), while others reported that fertilization/cleavage rates in older women were similar to that in younger women (Hull et al., 1996; Janny and Menezo, 1996; Lim and Tsakok, 1997; Klein and Sauer, 2001).

In the present study, fewer embryos were recovered from old Hereford-cross cows than their young daughters, consistent with the results of a study of Holstein cows (Lerner et al., 1986) in which the number of transferable embryos also decreased with advanced age. In an equine study (Carnevale and Ginther, 1995), fewer pregnancies were detected after intrafallopian transfer of oocytes from old versus young mares into young

recipient mares (detected by ultrasonography 12 days after transfer and insemination). Interestingly, however, the proportion of Grade 1 and 2 embryos, out of the total number of embryos recovered in the present study, did not differ between old and young cows. As well, the survival of embryos obtained from old cows and their young daughters after transfer (as singletons or pairs) into young recipients did not differ between age groups. Similarly, age did not influence pregnancy rate after embryo transfer in Holstein cattle (Lerner et al., 1986). On the contrary, the implantation/pregnancy rate in women after embryo transfer decreased with age of the embryo donor (Hull et al., 1996; Lim and Tsakok, 1997). However, it is common practice to transfer embryos immediately after cleavage in women (i.e., not cultured to blastocyst stage) (Blake et al., 2005). In the present study, pregnancy loss did not differ between embryos transferred from old versus young cows.

The hypothesis that developmental competence of oocytes declines with maternal age was supported; a lower proportion of embryos and a higher proportion of oocytes/uncleaved zygotes were recovered from old cows compared to their young daughters. Failure of fertilization or cleavage suggests that development is impaired at a very early stage in oocytes derived from old cows. Although fewer embryos were produced from the old cows, the proportion of good quality embryos recovered and the survival of good quality embryos after transfer into young recipients was similar between age groups. In conclusion, fertilization/cleavage rates were lower in oocytes from old cows than in their young daughters, but those that reached morula/blastocyst stage of development had similar developmental potential.

CHAPTER 7

OOCYTE MATURATION AND CHROMOSOME ABNORMALITIES

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7.1 Introduction

Decline in fertility with advancing age has been observed in women during their natural reproductive cycles (Tietze, 1957; Menken et al., 1986; Chandra et al., 2005). The success rate of assisted reproductive technologies in women also decreased with age (Wright *et al.*, 2006), and was attributed to an inadequate response to ovarian stimulation, reduced fertilization or implantation rates, higher gestational attritions, and higher incidence of chromosomal abnormalities in oocyte (Schwartz and Mayaux, 1982; Hull et al., 1996; Dew et al., 1998; Pellestor et al., 2003; Spandorfer et al., 2004). Studies involving oocyte donation from younger to older women demonstrated that the age related decline in fertility is due to reduced developmental competence of oocyte, and the uterine receptivity is maintained in women of advanced age (Sauer, 1998). Oocytes from women of advanced age had higher incidence of whole chromosome or single chromatid non-disjunctions when compared to younger women (Pellestor *et al.*, 2003). A well characterized animal model is required to study age-associated decline in fertility, and to investigate mechanisms of oocyte chromosomal abnormalities in women of advanced age.

A bovine model to study ovarian function in women has been proposed (Adams and Pierson, 1995), and was the basis of the discovery of follicular waves in women (Baerwald et al., 2003a; b). We proposed to extend this model to study age-associated

subfertility in women (Malhi *et al.*, 2005). Age related follicular and endocrine changes during a natural interovulatory interval were documented in 13- to 14-yr-old cows, and were compared to their 1- to 4-yr-old young daughters (Malhi *et al.*, 2005). Old cows had a rise in circulating concentrations of FSH, but had fewer 4- to 5 mm follicles recruited into a follicular wave when compared to their young daughters (Malhi *et al.*, 2005). After superstimulation, old cows had fewer large follicles than their young daughters (Malhi *et al.*, 2006). During follicle wave synchronization, the response of hypothalamo-pituitary axis to a combined exogenous treatment of estradiol and progesterone was similar (i.e., a new follicular wave emerged about 4 days after treatment) in old cows and their young daughters (Malhi *et al.*, 2006). In another study using same groups of old cows and their young daughters (Chapter 6), all animals were superovulated with exogenous gonadotropins, and were artificially inseminated. Lower proportion of embryos and a higher proportion of unfertilized ova were recovered from old cows than their daughters suggesting possible compromised fertilization in old cows (Chapter 6).

Germ cells undergo meiosis to produce haploid gametes. Oocyte meiosis consists of two divisions resulting in the production of a haploid oocyte as well as first and second polar bodies. Meiotic division in female germ cells of most mammals begins in prenatal life, but is arrested at diplotene stage of prophase I (Mehlmann, 2005). During antral follicle development in postnatal life, the oocyte resumes meiosis in response to a preovulatory surge of luteinizing hormone, completes first meiotic division and progresses to metaphase of second meiotic division (Mehlmann, 2005), and is then fertilizable. Oocytes in metaphase of second meiotic division can be obtained 22 h after surge of luteinizing hormone (King *et al.*, 1986).

Based on the possibility of reduced fertilization rate in old cows in our previous study (Chapter 6), we decided to test the hypothesis that oocyte meiotic maturation is compromised in old cows. The abnormalities of oocyte chromosome numbers originating during first meiotic division were also studied in old and young cows based on previous observations of higher incidence of oocyte chromosome numbers abnormalities in women of advanced age (Pellestor *et al.*, 2003). Cattle used for the study were born and raised at Goodale research farm, University of Saskatchewan. In an early herd-based study, about half of the cows were infertile by 13 year of age (Erickson *et al.*, 1976). In our herd, cows that failed to produce a calf annually were systematically culled. Thus 13- to 16-yr-old cows used in the present study were selected for fertility. Data from old cows were compared with their young daughters to minimize genetic variations.

7.2 Materials and Methods

The experimental protocol was approved by the University Committee on Animal Care and Supply under guidelines of the Canadian Council on Animal Care. The experiment was conducted on groups of crossbred Hereford cows (14 to 17 year old, n = 11) and their young daughters (3-7 year old, n = 12). All animals were born and raised at the Goodale Research Farm, University of Saskatchewan, Saskatoon, SK, Canada, and were maintained in a single outdoor corral. All animals were non-pregnant, non-lactating, and each had a corpus luteum detectable by ultrasonography at the beginning of this study. The experiment was conducted in replicates, but mother-daughter pairs were kept in the same replicate to minimize variations.

7.2.1 Ovarian Superstimulation

Ovulation was induced in all animals with two treatments of a prostaglandinF2α analog, cloprostenol (Estrumate®, 500 μg per treatment, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) given intramuscularly in the morning and evening. Ovulation was detected by the disappearance of a large follicle recorded during a previous ultrasound examination. Five to eight days after ovulation, ultrasound guided ovarian follicle ablation of all ≥5 mm follicles (Bergfelt *et al.*, 1994) was performed to induce the emergence of a new follicular wave. Superstimulation was initiated 24 h after follicle ablation i.e., before the expected emergence of a new follicular wave (Bergfelt et al., 1997). Superstimulation was performed using a standard FSH treatment protocol (Mapletoft et al., 2002; Malhi et al., 2006). Cows were given a total dose of 44 mg NIH-FSH-P1 FSH/100 kg body weight im divided bid over 3.5 days (i.e. 7 treatments of 6.25 mg Folltropin-V®/100 kg body weight; Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). Luteolysis was induced with two treatments of cloprostenol (Estrumate®, 500 μg per treatment) given intramuscularly 12 and 24 h after last FSH treatment. All cows were given an ovulatory dose of 25 mg Armour standard pLH (Lutropin-V®, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) 32 h after first treatment of cloprostenol. Ultrasound guided follicle aspirations of all follicles (≥5 mm) were performed about 18 to 20 h after LH treatment. Before follicle aspirations, the numbers of ≥6 mm diameter follicles were counted using transrectal ovarian ultrasonography (Malhi *et al.*, 2006).

7.2.2 Ultrasound Guided Ovarian Follicle Aspirations

Follicle aspirations were performed under caudal epidural anesthesia. The procedure of ovarian follicle aspiration was similar to follicle ablations as described previously (Bergfelt *et al.*, 1994). The follicular contents were aspirated into a custom-modified embryo filter (Filter Emcon, Cat. No. 7018, CDMV, Calgary, Alberta, Canada) containing 50 mL of Dulbecco's phosphate buffered saline 1X (Cat. No. 14040, Invitrogen, Burlington, Ontario, Canada) supplemented with 0.4 mL of heparin sodium (Hepalean®, 1000 U.S.P. units per mL, Organon Canada Ltd, Toronto, Ontario, Canada) and 0.1 mL of ET surfactant (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). A flow rate of 18 to 20 mL per min was maintained during follicle aspirations. The embryo filters were rinsed with Dulbecco's phosphate buffered saline into three petri dishes for each filter (Falcon 1001, Becton Dickinson Labware, Franklin Lakes, NJ, USA). The dishes were searched under stereo-microscope to recover cumulus-oocyte complexes (COC). The COC were evaluated and classified as described in Table 7.1.

7.2.3 In Vitro Maturation

The COC in the present study were obtained 18 to 20 h after LH treatment (in vivo maturation). Oocytes from cows with majority of expanded and compact COC were matured in vitro at 38.5°C with 5% CO_2 for 6 and 24 h respectively. Based on an early work (King *et al.*, 1986), the 6 h of in vitro maturation was given to allow a total time of 24 h for oocyte meiotic maturation. In vitro maturation for 24 h was given to COC to allow completion of first meiotic division, and to study oocyte chromosome numbers for detecting abnormalities of meiotic origin. In vitro maturation medium consisted of TCM-199 (Cat. No. 12340, Invitrogen, Burlington, Ontario, Canada) supplemented with 10%

heat-inactivated fetal bovine serum (Cat. No. 12484, Invitrogen, Burlington, Ontario, Canada), 25 μg/mL gentamicin, 0.2 mM pyruvate, 1 μg/mL estradiol (Cat. No. G1264, P5280, E2758 respectively, Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada), and 0.04 μg/mL FSH (Folltropin-V®, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). Cumulus cells surrounding the in vitro matured oocytes were removed by repeated pipetting in Dulbecco's phosphate buffered saline without calcium and magnesium (Cat. No. 14190, Invitrogen, Burlington, Ontario, Canada).

Table 7.1 Cumulus-oocyte complexes classification based on cumulus cell layer morphology

Number of cumulus cell layers	Cumulus cell morphology	Grade
≥ 2 layers	Full expansion	Expanded
≥ 2 layers	Compact	Compact
≤ 1 layer	Not assessed*	Denuded
≥ 2 layers	Expansion with dark clumps	Degenerating

* Insufficient cells to evaluate cumulus cell morphology

7.2.4 Preparation of Oocyte Chromosomal Spreads

Denuded oocytes from old cows and their young daughters were washed two times in Dulbecco's phosphate buffered saline. Oocytes were treated in hypotonic solution (0.8% sodium citrate for 8 min and subsequently in 0.075M potassium chloride for 5 min) and were fixed overnight in 3:1 methanol-acetic acid mixture. Each oocyte was placed on to an individual slide. Oocyte chromosomal spreads were prepared quickly by adding drops of 1:1 methanol-acetic acid on to the slides while blowing on to the slide at the same time. Oocyte chromosome spreads were stained with 8% solution of giemsa stain (Karyomax®, Cat. No. 10092, Invitrogen, Burlington, Ontario, Canada) in Gurr's buffer (Cat. No. 10582, Invitrogen, Burlington, Ontario, Canada) for 10 min.

7.2.5 Oocyte Nuclear Stage and Abnormalities of Chromosome Numbers

Oocyte chromosome spreads were observed under light microscope to determine their meiotic stage, and their chromosomes were counted to determine the abnormalities of numbers. Metaphase II oocytes with overlapping chromosomes, scattered chromosomes and < 28 chromosomes were considered as uncountable, most likely due to improper fixation and spreading technique. Oocytes with chromosome number 28 and 29 were considered as hypohaploid. Oocytes with chromosome number 31 and 32 were considered as hyperhaploid.

7.2.6 Hormone Analysis

Blood samples were obtained by jugular venipuncture prior to follicle ablation, prior to treatment with cloprostenol (i.e., 12 h after last FSH treatment), and prior to treatment with exogenous LH (Malhi et al., 2005; Malhi et al., 2006). The plasma was harvested and stored at -20°C until analysis. Plasma concentrations of LH and

progesterone were estimated by radioimmunoassay (Malhi et al., 2005; Malhi et al., 2006). The LH assay had a minimum detection limit of 0.06 ng/mL with a standard curve ranging from 0.06 to 8 ng/mL. The intra-assay coefficient of variation for LH was 2% for low reference samples (mean, 0.44 ng/mL) and 4% for high reference samples (mean, 1.05 ng/mL), respectively. The solid phase radioimmunoassay for progesterone had a minimum detection limit of 0.1 ng/mL. The intra-assay coefficients of variation for low (mean, 1.47 ng/mL), medium (mean, 2.63 ng/mL) and high (mean, 14.99 ng/mL) reference sample were 6%, 7% and 3% respectively.

7.2.7 Data Analysis

The follicle count data from old cows and their young daughters were compared with Student's t-test. The proportional data on COC morphology, oocyte nuclear maturation and abnormalities of chromosome numbers from old and young cows were compared with Chi-square analysis or Fisher's Exact test using frequency procedure of SAS (Statistical Analysis System Learning Edition 2.0, SAS Institute Inc, Cary, North Carolina, USA). Plasma progesterone and LH concentrations were analyzed by analysis of variance for repeated measures using the mixed procedure of SAS.

7.3 Results

Circulating concentrations of progesterone and LH prior to follicle ablation (Day 0), prior to first treatment with prostaglandinF2α analog (Day 5), and prior to treatment with exogenous LH (Day 6) did not differ between old cows and their daughters (Fig. 7.1). The progesterone concentrations decreased after treatment with prostaglandinF2α analog i.e., from Day 5 to 6 while endogenous LH concentrations increased during this time interval (Fig. 7.1). After superstimulation, old cows had fewer ≥6 mm follicles than their young daughters (Table 7.2).

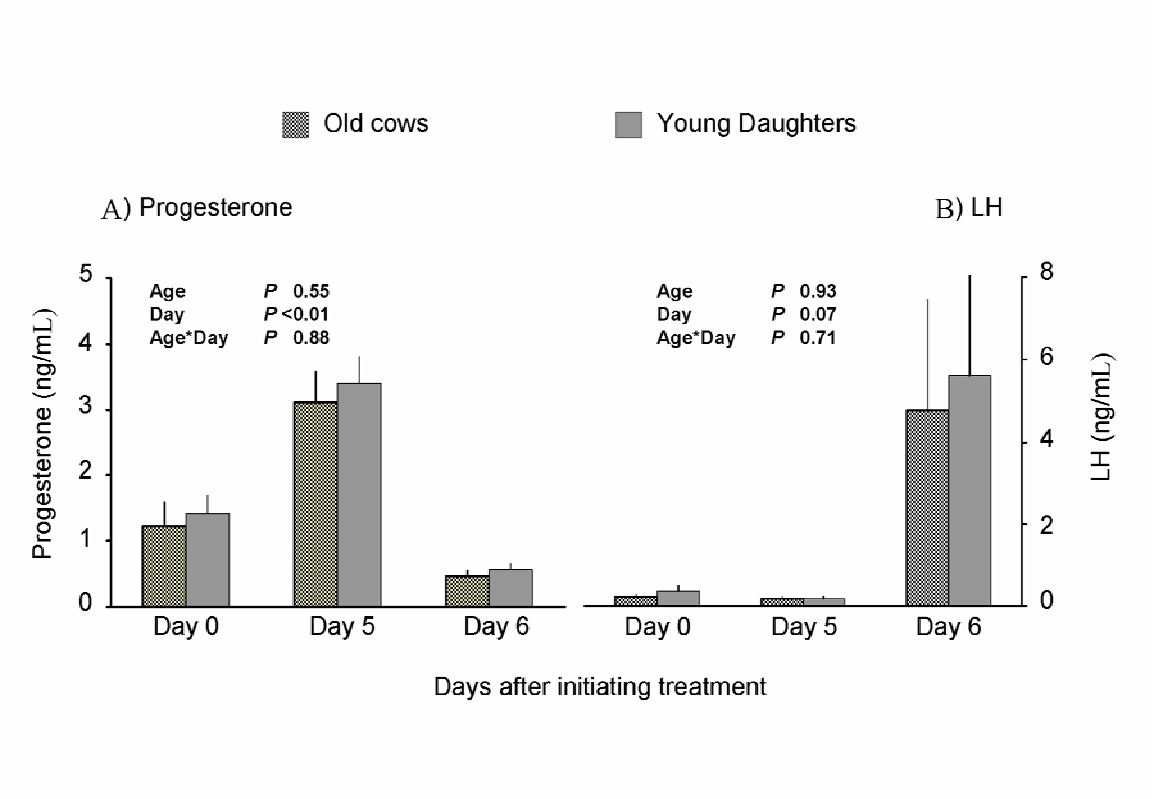

Figure 7.1 Concentrations (mean ± SEM) of progesterone (A) and LH (B) in old cows (n = 11) and their young daughters (n = 12) in plasma samples taken prior to follicle ablation (Day 0), prior to first treatment with cloprostenol (Day 5), and prior to treatment with exogenous LH (Day 6).

Table 7.2 Numbers of ovarian follicles (≥6mm) after superstimulation counted using ultrasonography and cumulus-oocyte complexes (COC) recovery by transvaginal ultrasound guided follicle aspirations in old cows and their young daughters

End Point	Old Cows (n=11)	Daughters (n=12)	*P*-Value
≥6mm follicles (mean ± SEM)	30 ± 3	47 ± 5	0.01
Total follicles aspirated	334	571	
Total COC recovered	214	350	
COC recovery (%)	64%	61%	

The percent recovery of COC after transvaginal ultrasound guided follicle aspirations did not differ between age groups (Table 7.2). Numbers of old cows and their young daughters with majority of expanded COC immediately after follicle aspirations did not differ (Old cows, 7 of 11 and Daughters, 9 of 12; P = 0.7). The rest of the cows in both age groups had majority of compact COC (Old cows, 4 of 11 and Daughters, 3 of 12).

The COC morphology in cows with majority of expanded COC immediately after follicle aspirations i.e., after in vivo maturation, and their oocyte nuclear stage after 6 h of in vitro maturation is summarized in Table 7.3. More than 90% of COC in both age groups had either expanded cumulus cell layers (Expanded) or did not have enough cumulus cell layers (Denuded) to convincingly assess cumulus layer expansion. Similarly, > 80% of oocytes in both age groups were in metaphase of second meiotic division (Metaphase II). A significantly (P = 0.02) higher numbers of oocyte in metaphase II were observed in old cows (119 of 129, 92%) than their daughters (124 of 150, 83%). Old cows had significantly more degenerating oocytes than their young daughters (Old cows, 9 of 155 and Daughters, 3 of 253; P = 0.01).

The COC morphology in cows with majority of compact COC immediately after follicle aspirations i.e., after in vivo maturation and their oocyte nuclear stage after 24 h of in vitro maturation is summarized in Table 7.4. More than 90% of COC in both age groups had either compact cumulus cell layers (Compact) or did not have enough cumulus cell layers (Denuded) to convincingly assess cumulus layer expansion. After 24 h of in vitro maturation, more than 95% of oocytes in both age groups were in metaphase II.

Table 7.3 Cumulus-oocyte complexes (COC) morphology and oocyte nuclear stage in old cows and their young daughters. The majority of COC had expanded cumulus cell layers

End Point	Old Cows (n=7)	Daughters (n=9)	*P*-Value
Numbers of COC evaluated	155	253	
COC morphology immediately after follicle aspirations i.e., after in vivo maturation			
Expanded	83/155 (54%)	93/253 (37%)	< 0.01
Compact	6/155 (4%)	9/253 (4%)	0.87
Denuded	57/155 (37%)	148/253 (59%)	< 0.01
Degenerating	9/155 (6%)	3/253 (1%)	0.01
Oocyte nuclear stage after in vivo maturation + 6 h of in vitro maturation			
Numbers of oocyte evaluated	129	150	
Metaphase II	119/129 (92%)	124/150 (83%)	0.02
GVBD* and Metaphase I	8/129 (6%)	24/150 (16%)	0.01
GV**	2/129 (2%)	2/150 (1%)	1.00

* Germinal vesicle breakdown ** Germinal vesicle

Table 7.4 Cumulus-oocyte complexes (COC) morphology and oocyte nuclear stage in old cows and their young daughters. The majority of COC had compact cumulus cell layers

End Point	Old Cows (n=4)	Daughters (n=3)	*P*-Value
Numbers of COC evaluated	59	97	
COC morphology immediately after follicle aspirations i.e., after in vivo maturation			
Expanded	4/59 (7%)	2/97 (2%)	0.20
Compact	2/59 (3%)	34/97 (35%)	< 0.01
Denuded	52/59 (88%)	61/97 (63%)	< 0.01
Degenerating	1/59 (2%)	0/97 (0%)	0.38
Oocyte nuclear stage after in vivo maturation + 24 h of in vitro maturation			
Numbers of oocyte evaluated	44	63	
Metaphase II	43/44 (98%)	61/63 (97%)	1.00
Metaphase I	1/44(2%)	1/63 (2%)	1.00
GV*	0/44 (0%)	1/63 (2%)	1.00

* Germinal vesicle

Oocyte chromosome numbers data from old cows and their young daughters has been summarized in Table 7.5. More than 50% of oocytes in both age groups had normal chromosome complement (i.e., chromosome number 30). When combined over age groups, 22 of 146 oocytes (15%) had diploid chromosome complement. Numerically, fewer numbers of old cows contributed to the total numbers of oocytes with diploid chromosome complement as compared to their young daughters (Old cows, 4 of 11 and daughters, 9 of 12; P = 0.10). Only 2 of 70 oocytes (3%) in old cows were hyperhaploid (chromosome number 31 and 32), and both were recovered from the same old cow. Numbers of hypohaploid oocytes (chromosome number 28 and 29) were significantly higher in young cows as compared to their mothers (Young daughters, 18 of 76 and Old cows, 7 of 70; P = 0.03).

Table 7.5 Abnormalities of oocyte chromosome numbers in old cows and their young daughters. All oocytes were in metaphase of second meiotic division

End Point	Old Cows	Daughters	*P*-Value
In vivo maturation + 6 h of in vitro maturation			
Numbers of countable spreads	46	53	
Chromosome number 30 (Normal)	37/46 (80%)	32/53 (60%)	0.03
Abnormalities of oocyte chromosome numbers			
Diploid	4/46 (9%)	13/53 (25%)	0.06
Chromosome number 31 to 32	2/46 (4%)	0/53 (0%)	0.21
Chromosome number 28 to 29	3/46 (7%)	8/53 (15%)	0.21
In vivo maturation + 24 h of in vitro maturation			
Numbers of countable spreads	24	23	
Chromosome number 30 (Normal)	16/24 (67%)	12/23 (52%)	0.31
Abnormalities of oocyte chromosome numbers			
Diploid	4/24 (17%)	1/23 (4%)	0.17
Chromosome number 28 to 29	4/24 (17%)	10/23 (44%)	0.06
Data combined for in vivo maturation + 6 h and 24 h of in vitro maturation			
Numbers of countable spreads	70	76	
Chromosome number 30 (Normal)	53/70 (76%)	44/76 (58%)	0.02
Diploid	8/70 (11%)	14/76 (18%)	0.24
Chromosome number 31 to 32	2/70 (3%)	0/76 (0%)	0.23
Chromosome number 28 to 29	7/70 (10%)	18/76 (24%)	0.03

7.4 Discussion

The effects of advanced age on oocyte meiotic maturation as well as on abnormalities of oocyte chromosome numbers were studied in old and young cows based on previous observations of impaired fertilization in older cows (Chapter 6), and higher abnormalities of chromosome numbers in oocytes obtained from women of advanced age (Pellestor *et al.*, 2003). We proposed to use the bovine model for the study of oocyte-associated subfertility in women of advanced age (Malhi *et al.*, 2005). Follicular wave emergence, selection and ovulation of a single follicle in a natural reproductive cycle were fundamentally similar between cattle and women (Adams et al., 1992b; Adams and Pierson, 1995; Baerwald et al., 2003a; b). A rise in circulating concentrations of FSH was observed in 13 to 14 year old cattle (Malhi *et al.*, 2005) similar to that reported in women of advanced age (Klein et al., 1996a).

The emergence of a new follicular wave after exogenous steroid treatment was synchronized in old cows and their young daughters (Malhi *et al.*, 2006). Fewer small ovarian follicles at the time of follicle wave emergence, and fewer large follicles after ovarian superstimulation were also observed in the same group of aged cattle when compared to their young daughters (Malhi et al., 2005; Malhi et al., 2006). In a subsequent study involving superovulation, artificial inseminations, embryo recovery and subsequent transfers into another group of young recipients (Chapter 6), lower proportion of embryos and higher proportion of unfertilized oocytes and/or uncleaved zygotes were recovered from old cows than their daughters suggesting compromised fertilization or gamete transport in old cows. Old cows used for these studies including present study were selected for fertility i.e., other cows in our herd that failed to produce a calf annually

were culled. The effect of genetic variations was minimized by comparing old cows with their younger daughters.

The meiotic maturation of an oocyte refers to its ability to resume meiosis in response to preovulatory surge of luteinizing hormone, complete first meiotic division, and progress to metaphase of second meiotic division. The present experiment was designed to test the hypothesis that oocyte meiotic maturation is compromised in old cows. To test our stated hypothesis, proportions of oocyte reaching metaphase of second meiotic division (after in vivo as well as in vitro maturation) were compared between old cows and their young daughters. Contrary to our hypothesis, old cows had similar (after in vivo maturation + 24 h of in vitro maturation) or significantly higher (after in vivo maturation + 6 h of in vitro maturation) numbers of oocytes in metaphase II than their daughters suggesting that reduced fertilization rate observed in a previous study (Chapter 6) was not due to compromised oocyte meiotic maturation. In the present study, old cows had significantly higher number of degenerating oocytes than their daughters. Similarly in a previous study (Aguilar, 2002), aged mares (> 15 year old) had higher proportion (47% versus 19% respectively) of oocytes with fragmented cytoplasm than younger mares (3 to 8 years old).

Oocytes obtained from old and young cows were also evaluated for their chromosome numbers. Higher incidence of whole chromosome and single chromatid non-disjunctions was reported in women of advanced age resulting in hypo- and hyperhaploid oocytes (Pellestor *et al.*, 2003). More than half of the oocytes evaluated for their chromosome complement in the present study were normal. The incidence of diploidy (15%) in oocyte chromosome in our study was higher than in previous reports (3

to 12%) on cattle oocytes (Slimane-Bureau and King, 2002). Proportion of oocytes with diploid chromosomal complement did not differ between old and young cows. We expected higher chromosomal anomalies in oocytes of old cows based on assisted reproductive technology data in women of advance age (Pellestor *et al.*, 2003). Conversely, the incidence of hypohaploidy was significantly lower in old cows as compared to their young daughters whereas hyperhaploidy was detected in only two oocytes, both obtained from the same old cow.

To conclude, our hypothesis of compromised oocyte meiotic maturation was not supported as indicated by similar proportion of metaphase II oocytes recovered from old and young cows. We did not observe higher abnormalities of chromosomal numbers in oocytes obtained from our group of highly fertile old cows when compared to their young daughters as indicated by the similar proportion of oocytes with diploid chromosomal complement.

CHAPTER 8

GENERAL DISCUSSION

An increasing number of women in North America delay childbearing until over 30 years of age (ASRM, 2004). Demographic studies on both early as well as contemporary human populations documented an age-associated decline in female fertility especially after 35 years of age (Tietze, 1957; Menken et al., 1986; Chandra et al., 2005). Moreover, the success rates of assisted reproductive technologies (i.e., donor insemination, in vitro fertilization and embryo transfer) in women also decreased with age (Schwartz and Mayaux, 1982; Hull et al., 1996; Dew et al., 1998; Wright et al., 2006). The success or failure of an assisted reproductive cycle has emotional, economic and health (i.e., neonatal and maternal) implications.

The mechanisms involved in the age-related decline in fertility are not well understood. In a natural reproductive cycle, higher circulating concentrations of FSH have been observed with advancing age in women (Klein et al., 1996a; Soules et al., 2001), but the effects of FSH (if any) on fertility have not been established. Direct comparisons of assisted reproduction data between older and younger women (Hull et al., 1996; Spandorfer et al., 2004) documented: 1) recovery of fewer oocytes in older women after ovarian superstimulation, 2) greater incidence of chromosomal numbers abnormalities (i.e., whole chromosome and single chromatid non-disjunctions) in oocytes and embryos recovered from older patients, and 3) lower embryo implantation rate after IVF-ET and higher gestational attrition with age.

Although not critically tested, the recovery of fewer oocytes after ovarian superstimulation in older women may be a consequence of decreased ovarian follicular reserve or reduced sensitivity to FSH. It was speculated that lower implantation rates and higher gestational attrition rates in older women may be due to factors intrinsic to the oocyte or due to an inadequate luteal or uterine function (Hull et al., 1996). However, oocyte donations from younger to older women suggested that luteal and uterine functions are maintained in women of advanced age, and the oocyte is primarily responsible for the age-related decline in fertility (Sauer, 1998). The decline in oocyte developmental competence may be a result of long-term accumulated damage or may be due to a recent imbalance of hormonal milieu or follicular growth factor system. It was speculated that mitochondrial dysfunction, accumulation of reactive oxygen species, DNA damage, spindle defects and shorter telomeres may be responsible for the decline in oocyte developmental competence with advancing age (Tarin et al., 2001; Thouas et al., 2005; Trounson, 2006). In addition, the numbers of apoptotic granulosa cells in preovulatory follicles were reported to increase with maternal aging (Sadraie et al., 2000). These hypotheses require critical testing.

It is difficult to get ethical approval for conducting basic research on human reproduction. In women, most of the reproductive research is being conducted on oocytes and embryos that failed to develop during an assisted reproductive cycle. Thus a well characterized animal model is required 1) to elucidate the cellular and the molecular mechanisms of an age-associated decline in female fertility, and 2) to develop and test strategies to improve fertility in women in their last childbearing decade. The desirable characteristics of an ideal animal model are 1) close phylogenetic relationship to humans,

2) long reproductive life span, 3) well described reproductive physiology with mechanisms similar to that described in women, 4) age-related decline in fertility as reported in women of advanced age, 5) well developed reproductive and molecular technologies as well as the ability to manipulate follicular development, 6) relatively fewer ethical issues for conducting hypothesis-based observational or interventional studies, 7) economical, 8) easy availability of older individuals, 9) ease of animal handling and data collection.

Primates such as rhesus monkey are probably the most suitable choice as animal models as they have a close phylogenetic relationship to humans, and females of this species experience menopause similar to that observed in women (Bellino and Wise, 2003). However, reproductive techniques or in vitro tools to manipulate oocytes/embryos are not well developed for this species. A variety of mouse models (surgical, gene knockout, chemical induced; reviewed in section 1.6.2) were proposed to study reproductive aging (Yuan et al., 2005; Danilovich and Ram Sairam, 2006). Oocytes and embryos could be easily obtained from mice under different treatment regimens for the purposes of manipulative studies. Moreover, techniques to produce loss or gain of function genetic mutations are well established in mice. Conversely, mice have a shorter reproductive life span unlike humans, and reproductive tissues are thus not exposed to long-term environmental insults. The mouse is a polyovular species (i.e., releases multiple oocytes during each reproductive cycle), and thus fundamental ovarian physiology may be different from monovular species (i.e., humans). Moreover, the detailed knowledge of ovarian follicular dynamics is lacking in both primates and mouse. The mare was also proposed as an animal model for the study of reproductive events in

women (Ginther et al., 2004) based on the fundamental similarities in ovarian follicular development of these two species. However, it is difficult to precisely control ovarian follicular development in mares, and assisted reproductive technologies (i.e., in vitro fertilization and embryo culture) are not very successful in this species.

We proposed a bovine model for the study of oocyte-associated subfertility in women of advanced age (Malhi et al., 2005). Events of the prenatal ovarian development in cattle (i.e., formation of gonadal ridge and ovary, initiation of meiosis and formation of primordial follicles) are contemporaneous to the similar events in women (Erickson, 1966). Follicular and endocrine events of the normal reproductive cycle; i.e., follicular wave emergence, follicle selection and ovulation were fundamentally similar between cattle (Adams et al., 1992b; Adams and Pierson, 1995) and women (Baerwald et al., 2003b; a). The bovine model was the basis of the discovery of follicular waves in women (Baerwald et al., 2003b; a). Furthermore, assisted reproductive and molecular techniques have been well standardized for studying oocytes and embryos obtained from cows.

By design, we used 13-14 year old crossbred beef cows (n = 10) and their 1-4 year old daughters (n = 10) that were born and maintained on the same farm throughout their life span. The same groups of animals were repeatedly used for a series of experiments over the period of four years. Old cows used in the present study were selected for fertility as the animals in this herd that failed to produce a calf every year were systematically culled. The use of contemporaneous mother-daughter pairs allowed us to minimize the effects of genetic and environmental variations. In a previous herd-based report, 55% of the cows were infertile by 13 years of age (Erickson et al., 1976).

To characterize the bovine model, we studied the effects of age on 1) follicular, luteal, and endocrine characteristics in a natural reproductive cycle, 2) recruitment of follicles into a follicular wave, 3) hypothalamo-pituitary-ovarian axis and its responsiveness to gonadotropin treatment, 4) superstimulatory response to exogenous gonadotropin treatment, 5) superovulatory response and its characteristics, 6) oocyte developmental competence, 7) oocyte meiotic maturation, and 8) abnormalities of oocyte chromosome numbers originating during meiosis I.

In the present study (Chapter 3), the follicular wave pattern in a natural reproductive cycle was maintained in old cows, and was consistent with that observed in previous studies in heifers (Ginther et al., 1989a; Ginther et al., 1989b; Knopf et al., 1989; Singh and Adams, 1998; Singh et al., 1998; Singh and Adams, 2000) as well as in normal young women (Baerwald et al., 2003a). Based on existing human literature, we hypothesized that reproductive aging in cattle is associated with elevated circulating concentrations of gonadotropins and reduced concentrations of steroid hormones in a natural estrous cycle. Older cattle in the present studies had higher circulating concentrations of FSH than their young daughters in a natural follicular wave (Chapter 3) as well as in an induced follicular wave (Chapter 4), analogous to women approaching the later stages of their reproductive years (Klein et al., 1996a; Soules et al., 2001; Burger et al., 2002; Santoro et al., 2003).

Old cows with a 2-wave pattern (Chapter 3) had smaller ovulatory diameter of the dominant follicle than that of young cows, and this may be due to reduced numbers or sensitivity of gonadotropin receptors in follicles of aging ovaries. A tendency for smaller corpus luteum diameters as well as for lower circulating concentrations of progesterone

during the luteal phase was observed in old cows in the present study (Chapter 3), and may be a direct consequence of smaller ovulatory follicle diameter in old cows, similar to the findings in an earlier study (Bryner et al., 1990). In women, progesterone concentrations also decreased in menopause transition (Soules et al., 2001). The cause-and-effect relationship between low progesterone (Chapter 3) and subsequent age-related effects on follicular dynamics (Chapter 3), superstimulation (Chapter 4, 5) as well as on fertilization/cleavage/early embryonic development (Chapter 6) remains to be elucidated.

We hypothesized that higher FSH concentrations in old cows would result in greater follicular recruitment in successive waves. This hypothesis was also based on a presumed increased rate of follicle loss during menopause transition (Gosden and Faddy, 1994) as levels of FSH rise (Klein et al., 1996a; Soules et al., 2001; Burger et al., 2002; Santoro et al., 2003). Conversely, old cows had fewer 2-5 mm follicles recruited in a natural wave (Chapter 3) as well as in an induced wave (Chapter 4). Lesser recruitment of 2-5 mm follicles into a follicular wave despite elevated circulating concentrations of FSH in old cows may be a result of 1) smaller ovarian follicular reserve, 2) reduced numbers of granulosa cells in follicles, 3) reduced numbers of gonadotropin receptors per granulosa cell 4) impaired receptor-hormone binding, 5) reduced responsiveness of granulosa cell after receptor-hormone binding, or 6) changes in the intrinsic ovarian follicle growth factor systems. However, these hypotheses require critical testing. In this regard, an age-related reduction in binding of FSH to its receptors was demonstrated in FSH-R heterozygous and wild-type mice (Danilovich et al., 2002).

Ovarian cycles are frequently manipulated in both cattle and women to obtain multiple oocytes for the purposes of assisted reproduction (Dew et al., 1998; Bo et al.,

2002). Experiments were designed to examine the response of the aging hypothalamo-pituitary-ovarian axis to exogenous treatments for ovarian synchronization and superstimulation. Previously, it had been demonstrated that a single treatment with estradiol and progesterone had a negative feedback effect on the release of gonadotropins from the pituitary gland, and could effectively synchronize follicular wave emergence in young cows (Bo et al., 1994; Bo et al., 2000). We hypothesized that aging of the hypothalamo-pituitary axis in cattle is associated with decreased synchrony in FSH suppression after estradiol and progesterone treatment, with a subsequent decrease in synchrony of the FSH surge and follicular wave emergence. However, we observed (Chapter 4) that estradiol and progesterone treatment suppressed circulating FSH in both age groups for 36 h, and the intervals from treatment to the subsequent FSH peak (3.7 ± 0.2 d) and wave emergence (4.3 ± 0.3 d) were not different between old and young cows and were consistent with that of previous studies (Bo et al., 1994; Bo et al., 2002).

Although the preovulatory LH surge after estradiol treatment was delayed in old cows compared to young cows, we did not detect a difference between age groups in the interval to ovulation (Chapter 4) which may be a reflection of insufficiently frequent ultrasound examinations (24 h intervals). The discrepancy between intervals to the LH surge, and to ovulation, warrants re-examination with more frequent ultrasonographic monitoring. Overall, the effect of exogenous treatments for ovarian synchronization was identical between old and young cows, and ovarian synchronization could be safely used in old cows for future experiments.

We expected a reduced follicular and ovulatory response in old cows following exogenous gonadotropin treatments based on our observation that old cows had fewer 2-5

mm follicles recruited into a follicular wave than their young daughters (Chapter 3, 4). Indeed, old cows had significantly fewer 6-8 mm, 9-11 mm and ≥12 mm follicles after ovarian superstimulation than their young daughters (Chapter 5). The fewer number of follicles in old versus young cows was consistent with the tendency for a lower ovulation rate in older cows; on average, young cows had 8 more ovulations per cow than old cows (Chapter 5). Results were also supportive of other studies in which the antral follicle count (all follicles ≥2mm diameter) was highly predictive of the ovarian superstimulatory response in cattle (Singh et al., 2004) and in women (Ng et al., 2000).

The ovulatory response of superstimulated ovaries was similar between age groups as indicated by the proportion of ≥6 mm follicles that ovulated after exogenous LH treatment (Chapter 5). Follicular and ovulatory responses of individual animals to successive superstimulatory treatments were highly correlated similar to a previous report (Singh et al., 2004), where the number of 2-6 mm follicles at follicular wave emergence was significantly correlated with their number in successive waves.

Based on human literature, we hypothesized that aging in cattle is associated with reduced developmental competence of oocytes. To test this stated hypothesis (Chapter 6), the same groups of old and young cows were superovulated, artificially inseminated with the semen from a single bull, embryos were recovered by non-surgical uterine flushings, and all good quality embryos were transferred into a different group of young recipient cows. Data from old cows and their young daughters were analyzed by comparing 1) the actual number of recovered oocytes/embryos, 2) the proportion of total oocytes/embryos recovered in each age group, 3) the proportion of oocytes/embryos recovered from each

individual, and 4) the pregnancy rate and numbers of calves born after transfer of embryos recovered from old and young cows into an unrelated group of young recipients.

Fewer embryos and a higher proportion of unfertilized oocytes and/or uncleaved zygotes recovered from aged cattle, compared to their young daughters, suggested that fertilization or cleavage rates declined with age (Chapter 6). This conclusion was supported by the observation that of the total oocytes/embryos recovered per cow, significantly more old cows (10/15, 67%) produced <50% embryos compared to their young daughters (4/16; 25%). The decline in fertilization or cleavage rates with age may be due to cytoplasmic or nuclear changes in an oocyte, and it needs further investigation.

Although the present experiment (Chapter 6) was not designed to directly test differences in gamete transport between age groups, oviductal transport of oocytes/embryos was similar (P=0.11) between age groups based on the number of oocytes/embryos recovered as a proportion of detected ovulations (66±6% in old cows and 53±6% in young daughters). Analysis of oocyte/embryo recovery by including data from only those animals that produced at least two or more embryos (i.e., confirming the presence of spermatozoa in the oviducts) revealed a higher (P=0.05) proportion of oocytes and uncleaved zygotes in old cows (50±10%, n=10) than in their daughters (28±5%, n=13). However, a more comprehensive study should be designed to test gamete transport in old cows. On a different perspective; fertilization, cleavage and oocyte developmental competence in old cows may be tested in an in-vitro system to circumvent issues related to gamete transport. The survival of embryos (morula/blastocyst) obtained from old cows and their young daughters after transfer (as singletons or pairs) into young

recipients did not differ between age groups (Chapter 6). The pregnancy loss also did not differ between embryos transferred from old versus young cows.

Based on the possibility of impaired fertilization in old cows (Chapter 6), the next experiment (Chapter 7) was designed to test the hypothesis that oocyte meiotic maturation is compromised in old cows. The meiotic maturation of an oocyte refers to its ability to resume meiosis in response to preovulatory surge of luteinizing hormone, complete first meiotic division, and progress to metaphase of second meiotic division. To test our stated hypothesis, proportions of oocytes reaching metaphase of second meiotic division (after in vivo as well as in vitro maturation) were compared between old cows and their young daughters. Contrary to our hypothesis, old cows had similar (after in vivo maturation + 24 h of in vitro maturation) or significantly higher (after in vivo maturation + 6 h of in vitro maturation) numbers of oocytes in metaphase II than their daughters suggesting that reduced fertilization rate observed in a previous study (Chapter 6) was not due to compromised oocyte meiotic maturation.

Oocytes obtained from old and young cows were also evaluated for their chromosome numbers (Chapter 7). We expected higher anomalies of oocyte chromosomal numbers in old cows based on data from women over 30 years of age using assisted reproductive technologies (Pellestor et al., 2003). More than half of the oocytes evaluated for their chromosome complement in the present study were normal. Proportion of oocytes with diploid chromosomal complement did not differ between old and young cows.

Based on consistent age-related effects on ovarian function, the bovine model may be as useful for the study of oocyte-associated subfertility in humans as it has been

for elucidating follicle dynamics during the natural menstrual cycle. In our group of highly fertile old cows, we did not detect an age-related increase in abnormalities of oocyte chromosome numbers. Observed differences in ovarian function between old and young cows may be expected to be a conservative estimate of changes occurring during the transition to reproductive senescence. The bovine model may be particularly useful for addressing issues relevant to age-related infertility in women such as 1) test of the hypothesis that antral follicle count is an accurate predictor of ovarian follicle reserve, 2) study of nuclear and cytoplasmic changes in the oocyte associated with subfertility, and mechanisms associated with chromosomal aberrations, 3) identification of oocyte or granulosa cell markers of fertility, 4) development of interventional strategies for improving ovarian stimulation and oocyte competence, and 5) elucidation of the role of telomere length and telomerase activity in aging somatic and reproductive tissues. However, it is definitely more expensive animal model than the mouse. I will conclude with the following quote (Austad, 1997):

> Many of the greatest leaps of understanding in biology (including *Theory of Natural Selection* by Charles Darwin) have come from comparative analyses of species rather than from the detailed examination of individual species. Comparative perspective needs to be resuscitated in experimental aging research, and that the comparative perspective is equally valid and important whether that research is taking place at the level of the individual, the organ, the cell, or the gene.

CHAPTER 9

GENERAL CONCLUSIONS

9.1 Follicular, Luteal and Endocrine Characteristics

The comparison of endocrine, follicular and luteal characteristics between 13-14 year old cows and their 1-4 year old young daughters revealed:

1. The follicular wave pattern was maintained in old cows.
2. Each follicular wave was preceded by a surge in circulating FSH.
3. Elevated circulating concentration of FSH in old cows.
4. Old cows had fewer follicles (4-5 mm) recruited into their follicular waves.
5. Circulating concentrations of LH or LH pulse frequency did not differ between age groups.
6. The ovulatory follicle had a smaller maximum diameter in old versus young cows.
7. Corpus luteum diameter tended to be smaller in old cows.
8. Luteal phase progesterone concentrations tended to be lower in old cows.
9. Old cows had higher circulating estradiol during preovulatory phase.

We concluded that changes in follicular dynamics and their endocrine control in old cows were similar to those previously reported in women approaching menopause.

9.2 Ovarian Synchronization and Superstimulation

To examine the effect of age on hypothalamo-pituitary-gonadal axis, old cows and their young daughters received exogenous treatments for ovarian synchronization and superstimulation, and their follicular and endocrine data were compared.

1. Elevated circulating concentrations of FSH in old cows and the FSH peak was temporally associated with the emergence of an induced follicular wave.
2. Estradiol and progesterone treatment suppressed circulating FSH in both age groups for 36 h. The intervals from steroid treatment to the subsequent FSH peak and wave emergence were not different between age groups.
3. The LH surge after estradiol treatment was delayed in old cows but no difference could be detected between age groups in the interval from estradiol treatment to ovulation.
4. Plasma concentrations of LH or the amplitude of the preovulatory LH surge did not differ between age groups.
5. During ovarian synchronization, old cows tended to have fewer 4 to 5 mm follicles at the emergence of an induced wave than young cows.
6. After ovarian superstimulation, old cows had 11 fewer large follicles (≥9 mm) 12 h after the last FSH treatment than young cows.

9.3 Superovulation

The present experiments were designed to study the effect of reproductive aging on recruitment of small (2-5 mm) antral follicles into follicular waves, and to compare follicular and ovulatory response in old cows and their young daughters.

1. Fewer 2-5 mm follicles were detected in old cows than in their young daughters at the expected time of follicular wave emergence.
2. Fewer ≥6 mm follicles were detected at the end of superstimulatory treatment in old cows than in their young daughters.
3. A tendency for a lower ovulation rate in older cows; on average, old cows had 8 less ovulations per cow than young cows.
4. The proportion of ≥6 mm follicles that ovulated was not different between age groups.
5. There was a highly positive correlation in the response of individual cows to successive superstimulatory treatments and the number of detected ovulations over years.

9.4 Oocyte Developmental Competence

To study oocyte developmental competence in advanced age, data from old cows and their young daughters were analyzed by comparing 1) the actual number of recovered oocytes/embryos, 2) the proportion of total oocytes/embryos recovered in each age group, and 3) the pregnancy rate and number of calves born after transfer of embryos recovered from old and young cows into an unrelated group of young recipients.

1. The total number of oocytes and embryos recovered did not differ between old cows and their daughters.
2. There were significantly higher mean number of oocytes and fewer mean number of embryos recovered from old cows than their daughters.
3. A higher proportion of unfertilized oocytes/uncleaved zygotes and a lower proportion of embryos were recovered from old cows than their young daughters.
4. The recovery of fewer embryos and a higher proportion of oocytes collected from aged cattle, compared to their young daughters, suggest that fertilization or cleavage rates decline with age.
5. Among the recovered embryos, the proportion of Grades 1 and 2 embryos did not differ between old cows and their young daughters.
6. The survival of embryos (morula or blastocyst) from old and young cows after transfer into an unrelated group of young recipients did not differ.
7. The survival of embryos transferred into recipient cows as singletons versus pairs also did not differ.

9.5 Oocyte Meiotic Maturation and Abnormalities of Chromosomal Numbers

Oocyte meiotic maturation and abnormalities of oocyte chromosome numbers were compared between old and young cows.

1. The percent recovery of cumulus oocyte complexes after transvaginal ultrasound guided follicle aspirations did not differ between age groups.
2. Numbers of old cows and their young daughters with majority of expanded COC after in vivo maturation did not differ.
3. More than 80% of oocytes in both age groups were in metaphase of second meiotic division.
4. Old cows did not have lower proportion of metaphase II oocytes than young cows.
5. More than half of the oocytes evaluated for their chromosome complement in the present study were normal.
6. The proportion of oocytes with diploid chromosomal complement did not differ between old and young cows.

CHAPTER 10

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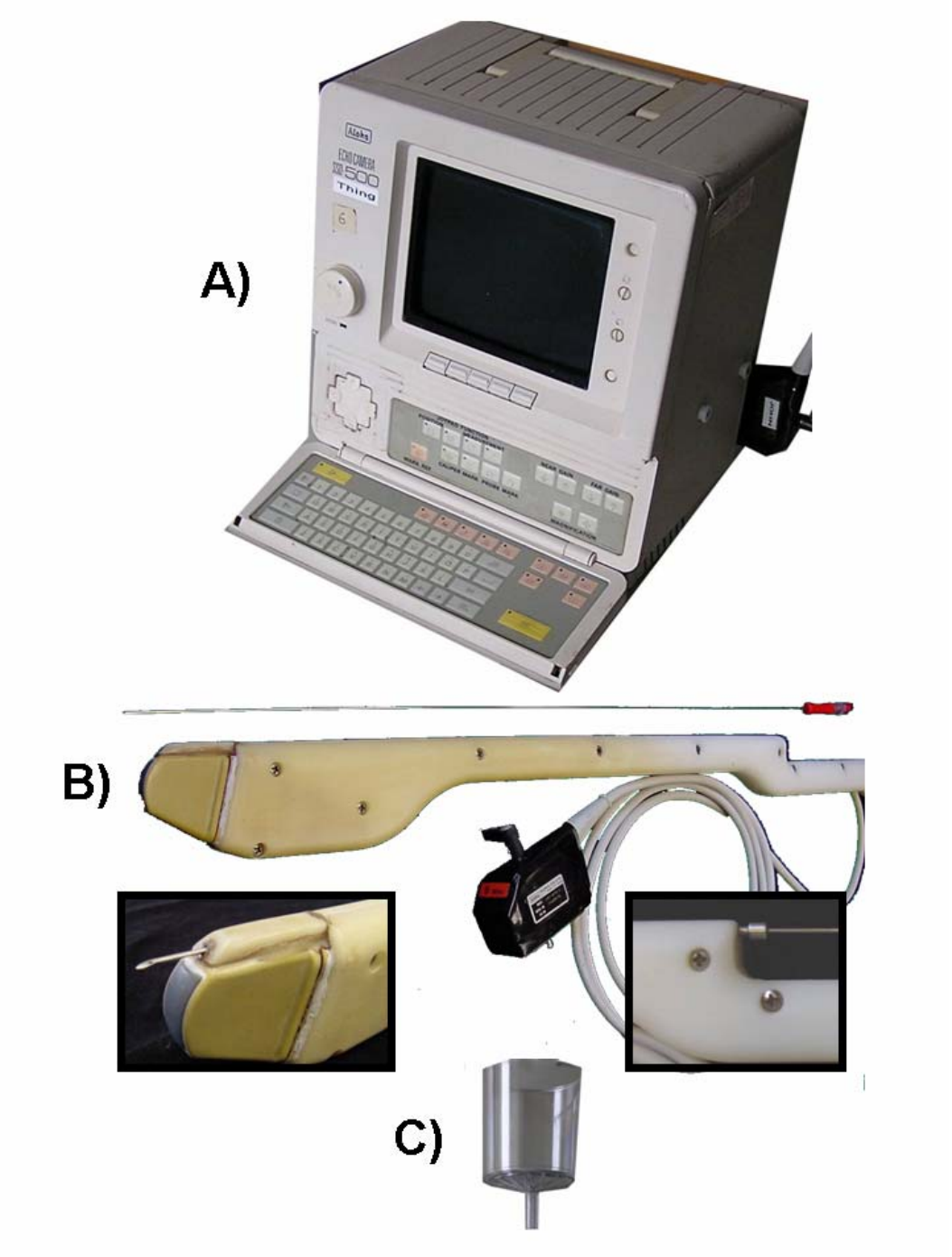
APPENDIX A

Equipment for Transvaginal Ultrasound Guided Follicle Aspirations

A) B-mode ultrasound scanner Aloka SSD-500

B) 5 MHz convex-array transducer fitted on a customized handle

C) Emcon embryo filter



APPENDIX B

A-B Cumulus-oocyte complexes morphology

C-D Stages of oocyte meiotic maturation

E-F Oocyte chromosomal spreads

G-H Stages of embryo development